

Crunch-time for Britain in CERN

Whether Britain will remain a member of the European Centre for Nuclear Research should be clarified at next week's council meeting. The time has come to stop playing chicken!

BRITISH discontents about continued membership of CERN, Europe's high-energy physics laboratory at Geneva, have been well advertised over the past several years, but are still far from clear. To be sure, the Science and Engineering Research Council (SERC), which pays the British membership subscription, has been increasingly embarrassed by the issue for at least the past decade, first because of the unpredictable inter-annual fluctuations of the value of sterling and thus of the money it must pay over and then, more recently, by the decline of its own disposable income relative to those of the equivalent organizations in other member states, which has made high-energy physics seem an uncomfortably large proportion of the funds available for supporting basic research. This year, the membership subscription alone will be \$58 million, more than a sixth of SERC's budget (quite apart from the extra sums that must be spent on the support of British groups planning to make use of CERN's facilities). It is not difficult to see why the Kendrew Committee appointed to look into the question held that this is too large a fraction of the funds available in Britain for basic research, a view endorsed by the Advisory Council for the Research Councils but not, significantly, by SERC itself.

Withdrawal

Next week (on Thursday and Friday), British delegates to the regular end-of-year meeting of the CERN council may or may not tell their fellow-delegates whether they have been instructed to give the year's notice of their intention to withdraw from the organization at the beginning of 1989 (or some later date), as required by CERN's constitution. By all accounts, the British government had not decided earlier this week what (if anything) it would do. Much, no doubt, will depend on the final version of the report on CERN's budget and administration by a group headed by Professor Anatole Abragam, established in response to British demands for economies at Geneva, which will be presented at the same council meeting. There are rumours that Abragam may have found ways in which CERN's budget could be reduced by the 15 per cent or so for which the British government has been asking, but the other members of CERN will not have been able to decide, by the time of next week's meeting, which (if any) of these proposed economies are acceptable. That will be decided only at the council meeting planned for next February.

On procedural grounds, Britain is in an awkward fix. There is a gentleman's agreement, reached in 1981, that no member of CERN would withdraw during the construction of the electron accelerator called LEP, due to be completed early in 1989. Although SERC, titularly autonomous, pays the bills, the British government has made it clear that a decision to withdraw from CERN would be political (which it is), and would not be left to the research council. Because it is unthinkable that Britain would go back on the LEP undertaking, the soonest that a British withdrawal could be organized would be early in 1989, requiring the payment of next year's membership fee and part of that for the succeeding year as well, but even to accomplish that, the British would have to give notice in writing before the end of this month, or before next February's consideration of the

Abragam report, prepared exclusively at their instigation, on which they would have very little influence if they had already given notice. This, no doubt, has inspired the shabby suggestion that the British government may give provisional notice next week, saying that the notice would be withdrawn if February's meeting should yield satisfactory economies (which would itself require the consent of the other members).

Misunderstandings

The plain truth is that Britain and its European partners are in danger of stumbling into a profound misunderstanding on this and many other issues of European collaboration on research. In many ways, the British position is the more seriously misunderstood. British opposition earlier this year to the European Community's research programme, originally, but in the event, timorously supported by West Germany, was more cogent than the proposed programme, and may have commanded greater sympathy if it had been advanced more intelligently. British opposition to (and, eventually, dissociation from) the European Space Agency's programme of manned spaceflight is entirely justifiable; there are many better ways to spend that kind of money, as West German researchers are claiming (see p. 510). But these reasonable actions are widely but illogically interpreted on mainland Europe as a sign of British detachment from European causes. (Last weekend's failure of the European Community's meeting in Copenhagen does not much help, even if the British were not, on this occasion, the chief stumbling-block.) And there is general misunderstanding on mainland Europe of the reality of the difficulties in which SERC (and the other research councils) finds itself. Critics of the British position on CERN are in danger of blaming close colleagues for circumstances over which they have no control.

The British scientific community, but especially the high-energy physics community, is also in danger of misunderstanding the circumstances that have arisen. The British research budget has been 'protected' against inflation since 1980, as the Prime Minister then promised, and has even been increased a little above the odds, but comparable research budgets in France, Italy and West Germany have been increased in real terms over the same period, in some cases substantially. Moreover, within the nearly constant ceiling of British research spending, increasing proportions of the funds available have been committed (at the instance of the government) to increased spending in previously neglected fields — more engineering research at universities, information technology and the like. SERC's freedom of manoeuvre has also been compromised by its failure radically to reduce its own internal spending on establishments which exist ostensibly to serve basic research in general, but which might in present circumstances be better dispersed among the institutions they are meant to serve. (Influent astronomers have recently been pointing out that the planned transfer of the Royal Greenwich Observatory to Cambridge, still hanging fire, should be taken as an opportunity for merging the Greenwich and Edinburgh Observatories; one benefit would be more money for research.) It would be easier simply to blame the British government for this catalogue of



troubles if the scientific community had been more vigilant in its defence of its own interests in the past eight years.

So what should be done about CERN? The British government, which has taken the decision into its own hands, had better move carefully. The decision not to participate in the European manned spaceflight programme may have been justifiable, but pulling out of CERN would be an entirely different matter even if the funds involved are smaller. For CERN has a unique place in the brief history of European collaboration. Quite apart from its accomplishments in high-energy physics as such, it has been one of the few models of a technique for international collaboration in which members' contributions are used to support the projects judged to be the most worthwhile, whatever the nationality of those who mount them.

No doubt CERN is expensive compared with what it would be elsewhere; it is, after all, mostly in Switzerland. No doubt there are also ways in which CERN's operations could be simplified and cheapened. But the British government will deserve all the scorn it will earn from other European governments if it plays its cards next week in a way that will mire CERN in a long wrangle about budgets, or in an artificial attempt to divide what is a unified programme of research into separable parts, with the intention then of opting out of some of them. Far better to pull out and shut up. Better still to agree to pay the subscription, but to make no promises about the degree to which British high-energy physics will be able to use the facilities at Geneva in the years immediately ahead. The reputation for niggardliness the British government has earned for itself in European research circles during recent years derives from its consistent failure to soften its habit of saying NO with an endorsement of the reasons for believing collaboration in research to be worthwhile; playing tactical games at next week's council meeting will be folly.

Either course would leave British high-energy physics in suspended animation, which would be ironical for the country in which Ernest Rutherford did most of his work. But that is the lesser evil. Indeed, it goes without saying that even if British membership of CERN continues, high-energy physics will win declining support until the research councils' own affairs are reorganized or until the government agrees that they should be compensated financially for the extra responsibilities they have undertaken in the past few years. It is natural that people who wish to play a part in the exciting developments that lie ahead in high-energy physics should be angered at the prospect that they may be excluded from them because their natural sponsors do not have the funds. But if that is how it emerges, they may the more effectively bend their energies to the improvement of the present state of affairs. □

US dollars for sale

The US government is occupied with the summit, but should also worry urgently about the dollar.

THE US administration, preoccupied this week with arms control, seems to have very little time or intellectual energy to spare for the more urgent crisis it should be tackling, the crumbling of the US dollar. Although the administration and the Congress did scramble together an outline scheme for reducing this year's budget deficit by the deadline of 20 November, the package was predictably unconvincing (see *Nature* 330, 193; 1987) to the international financial markets, not least because the Congress has yet to turn its agreement with the White House into legislation, and may yet renege on the deal. The consequence is that the value of the US dollar has fallen even further on the foreign exchanges. Since the beginning of this year, the average value of the dollar against the currencies of its trading partners has declined by 30 per cent, and would have fallen even further if the central banks of Japan and West Germany had not been buying dollars on the exchanges, incurring huge losses in the process. Yet the US administration shrinks from the inter-

national bankers' meeting which can alone bring stability to the system, partly from fear of congressional backsliding and partly because it wants to wait for some good news — perhaps an improvement of the trade imbalance — to suggest that the dollar has found its natural floor.

The danger in waiting for something to turn up is that it will accentuate the risk of a damaging upheaval in the world's economic system. The effects of the stock market crash beginning on 19 October have yet to become apparent in the United States, where the prospects of a recession will depend on the extent to which the inevitable but unknown reduction of domestic demand is counterbalanced by an increase of the export trade. The longer the uncertainty persists, the greater is the chance that the funds the Federal Reserve continues to pump into the US economy will show up in 1988 as inflation, with the uncomfortable consequences that will bring both in the United States and elsewhere, notably among the army of US creditors overseas. But the decline of the dollar has almost certainly gone far enough to give US exporters the extra edge they need to set the trade balance right. While there may be some in Washington who calculate that letting matters lie for a little while will soon persuade people elsewhere that cheap dollars are a good investment, that is too great a risk to take with the world's economy. It is just as likely that a continuation of the present state of affairs will merely force economic activity elsewhere to decline. It would be far preferable that the United States should do now what will at some point be necessary — to bridge the gap that lies ahead either by borrowing from its trading partners' central banks or by raising funds itself by selling debt denominated in yen and deutschmarks to the world at large. □

Academics should strike

Reasoned protests may be less effective than mass defection from government committees.

SHOULD British academics now go on strike? That is the question obviously prompted by one of the most explicit academic protests so far at the Education Reform Bill the British government published two weeks ago and plans to force through parliament. Last week Sir John Kingman, now vice-chancellor of the University of Bristol, but chairman of the Science and Engineering Research Council until 18 months ago, explained to his university court how the arrangements in the bill will destroy "once and for all, the present limited independence of the universities". He is right. The bill provides that the funds supplied to universities may have strings attached, and that the government may direct what those strings shall be (*Nature* 330, 299; 1987). If the government protests, in the debates that lie ahead, that it does not intend to use the provisions to undermine the residual independence of the universities, it should be asked why it has provided its successors with a means of doing just that.

Whether these provisions will be amended will depend on what happens to the bill in the House of Lords and, then, the House of Commons. Unless pure reason persuades the government that these provisions are mistaken, it can eventually insist on its chosen course. Curiously, however, the government will be relying throughout this period, and afterwards, on the willing services of British academics to help in administering British higher education, first preparing it for the slaughter and then arranging that the administration of the butchered corpse should be "cost-effective". What the government is asking is reminiscent of how the managers of concentration camps call on their intended victims for help. If British academics have any pride left (but the indignities of the past few years may have robbed them of it), they should now deny the government the help they need. A rapid thinning of the ranks of the University Grants Committee, destined for oblivion, would be appropriate, but there are many other government committees from which academics should promptly resign. □

NCI and Pasteur reveal details of AIDS foundations

- Eighty per cent of royalties go to research
- Human behaviour "must change"

Paris

THE Institut Pasteur and the US Department of Health and Human Services (HHS) last week put the official seal on a settlement agreement, announced in March this year, ending their dispute over ownership of the patent rights to the commercial blood test for antibodies to the virus causing AIDS (acquired immune deficiency syndrome) and over the claim to discovery of the virus (see *Nature* 326, 533; 1987).

Following the official signing at a ceremony at the Institut Pasteur in Paris on Friday, 4 December, Raymond Dedonder, director of the Pasteur and Robert Windom, US Assistant Secretary for Health, handed over cheques totalling \$3.7 million to endow two foundations — the French and American AIDS Foundation and its offspring, the World AIDS Foundation. The intention to set up a French and American AIDS Foundation was announced in Washington on 31 March this year by President Ronald Reagan and French Prime Minister Jacques Chirac.

Under the terms of the settlement agreement, each party agrees to donate to the French and American AIDS Foundation 80 per cent of royalties it receives from sales of the antibody screening kit, estimated at over \$4 million a year.

The foundation will fund international research into "the cause, detection, prevention, treatment and cure of AIDS", with 25 per cent going to research and educational projects benefiting developing countries. It will be directed by a board comprising three nominees from the HHS and three from the Institut Pasteur.

Luc Montagnier, of the viral oncology unit of the Institut Pasteur and Robert Gallo, of the US National Cancer Institute — who were at the centre of the patents dispute — used the occasion to issue a joint statement emphasizing their intention to "continue to share ideas, information and reagents" — a cooperation which, Gallo stresses, was not interrupted by the legal battle. For Montagnier, the formation of the French and American AIDS Foundation was "an extremely positive and happy conclusion" to the dispute.

Earlier this year, with encouragement from Jonas Salk, Montagnier and Gallo jointly authored a chronology of critical published findings leading to "the discovery and establishment of AIDS as a retroviral disease" (see *Nature* 326, 435;

1987). The chronology, supporting the "joint invention" of the AIDS virus antibody test kit, also formed part of the agreement legally executed in Paris last week, together with mutual licensing of the blood test — and of any improvements to it — and the co-signing of existing patent applications.

To facilitate the rapid sharing of results, a worldwide communications system, to be known as 'WIN', will be created. "This will become the basis of an international data bank of AIDS information, including fundamental and clinical research", said Gallo.

Lawyer Ira Millstein, of Weil, Gotshal and Manges, who acted for the Institut Pasteur in the patents dispute, announced the intention of the French and American AIDS Foundation to establish a non-governmental World AIDS Foundation, with a broader, more socially orientated remit. According to Millstein, "the precise priorities of the foundation will be decided by the board of governors" with policies intended to complement the work of the World Health Organisation and various governmental agencies "to develop public consensus over the health policies required to contain the spread of AIDS". Both Montagnier and Gallo are to be members of the board of governors. The World AIDS Foundation will be funded initially by the French and American AIDS Foundation, but will be expected to attract future support from independent fundraising.

Until a vaccine against AIDS is found, Millstein added, "containment of the disease will require change in human behaviour". He hoped that the World AIDS Foundation would play a role in ensuring that governments' health policies are based on "the best knowledge, information and medical judgements" and not on "misinformation, lack of knowledge or prejudice" — a view endorsed by French Health Minister Michèle Barzach.

The poetic justice of using money from sales of the antibody test-kit to fund research escaped neither Barzach nor Robert Robertson, General Counsel for the HHS and member of the World AIDS Foundation governing body. "In this way", said Robertson, "every time a unit of blood is tested, not only does the safety of the blood supply increase, but also the resources available for AIDS research grow".

Peter Coles

AIDS vaccine developments, see page 509.

AIDS report produced early

Washington

DESPITE controversy and ideological clashes, the US "Presidential Commission on the HIV Epidemic", named after the virus that causes AIDS, put out its preliminary report last week. The report identifies four key areas that the commission will focus on in the coming months: the accumulation of realistic data on the spread of the HIV virus in the United States, more home health care for AIDS patients, increased development of drugs to treat AIDS and the expansion of drug abuse treatment programmes.

The commission issued the report six days earlier than its 90-day deadline, despite the resignation of its chairman and vice-chairman two months ago over internal conflicts (see *Nature* 329, 570; 1987), and a lawsuit brought by public-interest groups claiming that the commission lacks ideological balance and members who are affected directly by AIDS. Critics also blame President Ronald Reagan for establishing the commission as a political solution to the AIDS problem that is not likely to work.

The poor performance of the commission has prompted a review of the Federal Advisory Committee Act — the act that authorizes the creation and governmental supervision of committees to advise the President — by the Senate Governmental Affairs Committee and an investigation of the commission by the General Accounting Office. The commission's new chairman, retired Admiral James D. Watson, testified before the Senate last week that although the commission had "no working strategy" when he first took over, it is now on track and working towards its goals.

The commission's final report and recommendations on the legal, ethical, social, medical and economic implications of AIDS is due next September. Watson says a series of interim reports on the four most critical topics will be issued in February.

At a news conference announcing the commission's report, the Secretary of Health and Human Services, Otis R. Bowen, also announced that the random blood-testing of the US population to determine the prevalence of HIV would be postponed. In the meantime, a 'family' of surveys based on anonymous blood samples taken at selected hospitals and clinics will be administered in the 20 cities with the highest incidence of AIDS, and in 10 other cities with a low to moderate number of AIDS cases. Bowen said that the previous estimate of 1 to 1.5 million people in the US infected with HIV is probably still valid.

Carol Ezzell

Research teams disperse as UK government rethinks Alvey

London

THE British government's strained efforts to formulate a long-term programme for information technology research appear to have ground to a complete halt amid a wide-ranging internal review at the Department of Trade and Industry (DTI). At present, Britain's information technology research drive is through the DTI-coordinated Alvey programme, launched in 1983 with £200 million of taxpayer's money (from the budgets of the DTI, the Science and Engineering Research Council and the Ministry of Defence) and £150 million from private industry. The programme was to last a nominal five years, although all the available funds have now been allocated.

Last July the under-secretary of state at the DTI, John Butcher, publicly pledged government support for a follow-up programme, but details have failed to emerge and researchers in the field are becoming increasingly dispirited over the government's apparent lack of interest. Further gloom will follow next month when a study is published claiming that the £70 million spent on Alvey's software engineering research has effectively been wasted, and that the programme as a whole was misconceived. The conclusions are at odds with most appraisals of the programme, which indicate that Alvey was on the whole a successful experiment.

Recent movements within the Alvey directorate suggest that if a follow-on programme is approved, its organization will be substantially different from that of its predecessor. In October, Alvey's director, Brian Oakley, retired; his place was officially taken last month by Timothy Walker, until then private secretary to Lord Young, the new minister at the department. At the same time, responsibility for the research side of the DTI's electronic applications division (which supports research into superconductors, opto-electronics, gallium arsenide and molecular electronics) was passed to the Alvey directorate, expanding its size by about half and drawing it into the overall machinery of the DTI. The department is evidently endeavouring to streamline the administration of its collaborative research programmes.

Draft copies of the report attacking the Alvey programme have infuriated many supporters of the programme, not least because it comes from the London Business School's Centre for Business Strategy (CBS), which had a brief but by all accounts acrimonious relationship with Alvey when it was contracted to evaluate various aspects of the programme. After two years, it became apparent that the

team and Alvey had different views on what comprised an evaluation, and CBS's official involvement ended. The new report, compiled by Peter Grindley, does not, therefore, have official Alvey status (as the programme's supporters are quick to point out), although it does draw on the information collected by the official evaluators (Grindley was not one of them). The report is an overview of the UK software industry, and touches only briefly on Alvey's role. Nevertheless, it is outspoken in its criticism.

Grindley attacks the programme for focusing on "systems factories", concentrating on software production and efficient coding. Such a policy, says Grindley, amounted to an expensive mistake. "The main opportunities are not in efficient coding but in the solution of broader, unstructured problems within a changing environment. What is needed is more flexibility, not more formalism."

Grindley questions the need for government intervention in software design techniques, arguing that the Alvey programme discouraged British companies from seeking overseas partners, insulating Britain and providing no formal mechanism for the international transfer of technology: "A game of nationalistic non-cooperation is one the UK is very poorly placed to win".

Alvey's official evaluators, from policy

research units at the universities of Manchester and Sussex, do not share Grindley's views. In their interim evaluation report, presented to the DTI in October, the teams concluded that Alvey had generally been successful, with a significant impact on academic research and an increase in collaboration between academic and industrial researchers.

The report stressed the need for a follow-up programme; the delay in an announcement of the government's intentions is already causing research teams to disintegrate. For the academic partners at least, the absence of a government policy is causing frustration and is impeding the formulation of long-term strategy.

A blueprint for an Alvey follow-on was submitted to the DTI in November last year, under the title "IT86" and compiled by a committee headed by Sir Austin Bide. That report recommended a collaborative programme similar to Alvey, but with emphasis on applications (the Alvey programme concentrated on 'pre-competitive' research). The government would provide £425 million, with industry's contribution bringing the total to around £1,000 million.

The government was expected to announce its intentions soon after the general election last June, but instead declared that a second committee would be set up to study Bide's proposals. It is suspected that the government is reluctant to provide such a large proportion of the total funding, given the 'near-market' nature of the envisaged programme.

Simon Hadlington

Japanese go-ahead for Cable and Wireless

Tokyo & London

A DETERMINED effort by Britain's Cable and Wireless to break into Japan's lucrative international telecommunications market has at last paid off. The Ministry of Post and Telecommunications has granted a licence to International Digital Communications (IDC), a consortium headed by the British company, to operate in competition with two other international telecom companies, the recently privatized monopoly Kokusai Denshin Denwa (KDD) and a rival consortium, International Telecommunications Japan (ITJ), led by giant Japanese trading companies.

The decision to grant IDC a licence ends a lengthy battle by Cable and Wireless which at one stage threatened to develop into a major trade dispute between Japan and the United Kingdom. The ministry tried to force a merger between IDC and ITJ which would have considerably diluted Cable and Wireless's shareholding. But the plan foundered on ITJ opposition to IDC's plan to lay a trans-Pacific optical fibre cable. The cable is a key link in Cable and Wireless's planned network of optical fibre

cables stretching around the globe that will compete with networks operated by KDD, American Telephone and Telegraph (AT&T) and British Telecom.

The ministry has said that it will grant IDC permission to lay the trans-Pacific cable, with a capacity equivalent to 11,000 telephone circuits, but the number of circuits licensed will be limited to prevent 'oversupply' in international telecommunications services — KDD and AT&T plan to lay two trans-Pacific optical fibre cables that will compete with the IDC cable (see *Nature* 328, 477; 1987). A spokesman for Cable and Wireless says there is "nothing out of the ordinary" about this condition, in fact it was "expected".

Both IDC and ITJ plan to begin services in the spring of 1989, offering rates at least 20 per cent cheaper than KDD. In addition to the trans-Pacific cable, which should be in place by 1989–90, IDC will lease circuits on Intelsat satellites, while ITJ will lease circuits on the satellites and a trans-Pacific cable (TPC-3) that will be laid by KDD and AT&T next year.

David Swinbanks & Simon Hadlington

Galileo takes interesting but indirect route to Jupiter

Washington

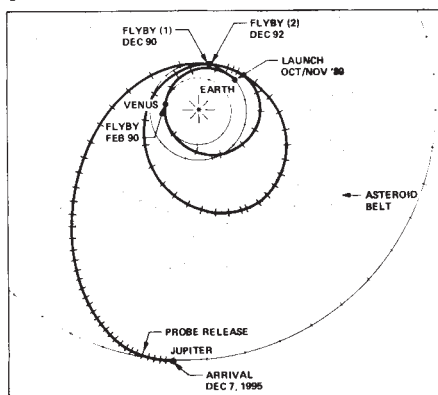
GALILEO, the most sophisticated planetary probe ever built, is currently sitting in pieces at the Jet Propulsion Laboratory (JPL) in Pasadena, California. But all that will change shortly. Mission scientists have determined the precise date it should arrive at Jupiter, and mission engineers will next month reassemble the craft, in preparation for its October 1989 launch.

Once, Galileo was a one-planet mission, but necessity has forced more elaborate plans. Cancellation of two different booster rockets has meant that there is no launch system at present in the US fleet that can place Galileo onto a direct trajectory for Jupiter. So last year, JPL scientists came up with a planetary tour dubbed VEEGA (for Venus, Earth, Earth-gravity assist) that will get Galileo to Jupiter in 6 years. The space shuttle *Discovery* will boost Galileo into Earth orbit, and an IUS (inertial upper stage) will start the craft on its planetary tour.

After launch, the spacecraft will fly towards the Sun, receiving a gravity boost from Venus in February 1990. Galileo will then return to Earth in December 1990 for a second gravity assist, going into a two-year solar orbit that will bring the spacecraft within 1,000 km of Gaspara, a type S asteroid 15 km in diameter. Arriving back at Earth in December 1992, Galileo will receive a third gravity assist to begin its journey to Jupiter.

Outward bound to Jupiter, Galileo will encounter Ida, another type S asteroid in August 1993. Five months before arriving at Jupiter, Galileo's planetary probe will be released (Galileo is really two spacecraft in one). The probe will follow a

ballistic trajectory into Jupiter's atmosphere at 6°N latitude. The Galileo orbiter will make a minor adjustment to its trajectory so it will orbit the planet, receiving yet another gravity assist from Io as it approaches the planet. The orbiter will orbit overhead for five minutes as the probe sends back data on its descent to the planet's surface.



Trajectory for the Galileo spacecraft. Subdivisions marked on each orbit represent 100 days for Jupiter, and 30 days for the spacecraft, Earth and Venus.

Galileo scientists intend to take advantage of its complicated trajectory. At Venus, for example, Galileo's infrared spectrometer will be used for studies of the atmospheric composition. During the Earth-Moon encounters, Galileo will be able to make near-infrared measurements of the far side of the Moon for the first time.

The change in trajectory has forced extensive modifications to the spacecraft, such as sunshades designed for sensitive instruments.

Joseph Palca

NASA space station contractors get the go-ahead

Washington

THE National Aeronautics and Space Administration (NASA) last week announced the winners of the race among aerospace companies for the initial large contracts to build a manned space station.

The largest single contract, estimated at \$1,900 million, went to McDonnell Douglas Aeronautics Co. to build the structural framework of the station. Rockwell International's Rocketdyne Division won the contract worth some \$1,600 million to develop the station's electrical power supply system, including solar power units, and Boeing Aeronautics will assemble a team to build the crew living quarters and laboratories at a cost of \$750 million. General Electric will

build a free-flying platform that will carry scientific instruments. That contract is estimated to be worth \$800 million.

All the companies involved in the race spent large sums of money in an attempt to win the lucrative contracts. The biggest loser was Martin Marietta Corporation, with no defined role in the project so far.

These contracts are for only the first phase of work that will be needed to place a working space station in orbit. The space station as at present conceived will cost some \$14,000 million to become operational. But congressional reticence to start new, expensive projects makes it almost certain that NASA's plans will have to be modified to accommodate fiscal realities.

Joseph Palca

Another AIDS vaccine

A SECOND vaccine against AIDS has been given clearance by the US Food and Drug Administration for testing in humans. The vaccine, developed by the Oncogen subsidiary of Bristol-Myers, consists of vaccinia virus containing the genes for the coat proteins for HIV-1.

The vaccine will be administered to between 30 and 60 HIV-negative homosexual volunteers, at the Pacific Medical Center in Seattle, Washington, to determine its safety and immunogenicity.

The first AIDS vaccine in the United States, developed by MicroGeneSys and the US National Institutes of Health, was approved for testing in humans in August. It is based on the coat protein gp160.

The efficacy of any vaccine is not determined until Phase III clinical trials. The vaccine developed by Oncogen has shown encouraging results in chimpanzees, however (*Nature* 328, 721-723; 1987). C.E.

Biogen patent

THE troubled biotechnology company Biogen has received a US patent for hepatitis-B antigens produced through genetic engineering that could be a significant source of revenue to the company, whose financial statements have been running deeply in the red. Biogen will not develop hepatitis-B products, but royalties from licensing the patent to producers of hepatitis-B vaccines and tests could earn them up to 10 per cent of the estimated \$200 million market for the products. C.E.

New NIMR director

Dr John Skehel (rhymes with flail) has been appointed director of the National Institute of Medical Research, Mill Hill — the largest of the UK Medical Research Council's laboratories.



A 46-year-old virologist, Skehel has worked at the institute since 1969 and has been head of one of its four divisions for the past two years. He takes over a post once held by Sir Peter Medawar from Dr Dai Rees, new secretary of the Medical Research Council. Skehel's research has concentrated on influenza viruses, particularly on the haemagglutinin protein. Recently, his attention has turned to the envelope protein of the human immunodeficiency virus. P.N.

MRC cold unit closes

BRITAIN'S Medical Research Council is to close its 40-year-old Common Cold Unit in Wiltshire when the unit's director, Dr David Tyrrell, retires in 1990. More than 18,000 volunteers have passed through the unit, whose annual budget is £500,000.

S.L.H.

More scientific contact between East and West Germany?

Munich

GLASNOST has arrived at the East German Academy of Sciences. Despite the danger of scientists defecting, the East German Academy has agreed in principle to increase scientific exchange between the two Germanies.

Academy president Werner Scheler made an unprecedented visit to Bonn on 2 December to meet his counterpart in the Deutsche Forschungsgemeinschaft (DFG), president Hubert Markl. Scheler invited Markl to East Berlin in the spring of 1988 to sign a final agreement.

The DFG, which supports much of the basic research in West Germany, has 29 such agreements with countries all over the world, including most of Eastern Europe. For example, the Soviet Union has been sending researchers to West Germany since the early 1960s, although the agreement between the DFG and the Soviet Academy of Sciences was signed only in 1970. All exchanges are financed

out of the DFG budget except those with the Soviet Union, which the Research and Technology Ministry supports.

Scheler came to Bonn only after a three-year delay in accepting the invitation of Markl's predecessor, Eugen Seibold. According to Eva-Maria Streier of the DFG, Scheler said he was able to visit only because of the current political thaw between East and West Germany. The East German delegation was uncharacteristically quick to accept the agreements between the DFG and other Eastern European countries as a basis for negotiating the new agreement. Even researchers in West Berlin may participate.

But large-scale exchange is unlikely, said Doris Schenk of the DFG, because of the risk that East German researchers might defect to West Germany. In the case of Soviet researchers, said Schenk, only 15–20 per cent of those specifically invited actually receive permission to visit West Germany.

Steven Dickman

Basic research is threatened by West Germany's space plans

Munich

FEAR has gripped the West German research community following the government's decision to participate in the three large projects of the European Space Agency (ESA).

In an unusually forceful statement, Heinz Staab, director of the Max Planck Gesellschaft (Max Planck Society or MPG), called upon the West German cabinet to finance its new space plans without damaging basic research. Staab said that there is "no sufficient scientific reason" to begin a European programme of manned space flight and that it is "absurd" to allow the financial pressures generated by the space programme and tax reform to reduce government support for basic research.

Staab's statement puts him on a collision course with Research and Technology Minister Heinz Riesenhuber. Riesenhuber has said that the three projects (the Hermes shuttle, the Columbus space station and the Ariane 5 launch vehicle) will cost West Germany DM500 million more a year than had been planned for the space programme. This money will come partly from the Research and Technology Ministry (BMFT) and partly from elsewhere in the West German budget.

BMFT will have about DM7,600 million at its disposal in 1988. The MPG accounts for about DM500 million of that, with the rest of its DM1,143 million bud-

get (in 1987) coming from the *Länder* and private contributions.

Historically, West Germany's big science organizations — the MPG, the Deutsche Forschungsgemeinschaft (DFG), and the Westdeutschen Rektorenkonferenz — have held their own in budgetary conflicts with the government. In 1984, for example, the leaders of these three organizations wrote a letter to the government defending their budgets in the face of a similar challenge from the space programme. Their request for additional funds was granted.

Staab says that big projects begin to operate "under their own impetus" and that they are always "much more expensive than predicted". As West Germany had agreed to support the ESA projects for other than scientific reasons, Staab said that the research budget should not have to suffer. He added that there is sufficient justification for unmanned space missions (such as the X-ray satellite ROSAT), which are also much less expensive.

Staab's views were echoed in a more muted way by Hubert Markl, president of the DFG. Markl has been travelling tirelessly through West Germany this autumn, defending basic research to anyone who will listen. The DFG's budget was increased by 3 per cent for 1988, which he says is far from sufficient.

Markl has called attention to the

Superphénix not to rise from ashes yet

Paris

THE French prototype 1,242-MW fast breeder reactor, Superphénix, at Creys-Malville near Lyons, will not start up before mid-1988, according to reports from the Ministry of Industry. The reactor was shut down last June, following the discovery of a leak in a steel storage chamber containing liquid sodium (see *Nature* 328, 100; 1987). When the chamber was drained, three 45-cm-long hairline fissures were found. The cause of the cracks has not yet been determined, but Electricité de France (EDF), which runs the reactor, argues that it could run safely for over a year without the defective chamber — which is used to store radioactive fuel on its way to and from the reactor core — while tests are carried out.

Industry Minister Alain Madelin, who is considering a confidential EDF report, expects that the reactor will nevertheless stay closed, at least until the middle of next year. Close to the Swiss border, the reactor has never been popular with France's neighbours, and restarting it without the chamber would be sure to bring howls of protest in a year of presidential elections. It is also not clear how fuel rods from the core would be stored in the event of a major accident.

Superphénix has already cost £2,000 million, making the electricity it produces 50 per cent more expensive than that from other French reactors. The prospect of a further bill of as much as £40–£50 million to repair or replace the storage chamber, putting the reactor out of action for perhaps three years, is a thorn in the side of Madelin, who, like his predecessors of both political colours, had hoped that Superphénix would be the first of a new generation of reactors in France.

The whole nuclear programme has slowed in France, since its peaks in 1977 and 1979 after the oil crisis. Instead of building one new reactor a year, as was the case from 1985 to 1987 — the last to open was at Nogent, near Paris, this year — there will be one new reactor every two years.

Peter Coles

"mountain of scientists" resulting from the "mountain of students" that has filled West German universities to overflowing for the past decade. "It would be stupid for us to stop supporting them now", he said.

Markl added that the universities could use some of the thousands of millions of deutschmarks that are now being freed to stimulate the West German economy. "We shouldn't just spend that money for building highways", he said, but, rather, "we should make it available to universities for investments in equipment that they desperately need". Steven Dickman

Talking war and peace at pre-summit Stanford meeting

Palo Alto

THE arrival of the Soviet leader, Mr Mikhail Gorbachev, in Washington this week was happily timed for Stanford University's Center for International Security and Arms Control. A long-planned conference on arms control, falling by chance on the last weekend before the summit, benefited from the participation of two of Gorbachev's scientific advisers. Their presence — by videolink from Washington — made up to some extent for the failure of Andrei Sakharov to win permission to attend.

The Soviet participants, academicians Yevgenii Primakov and Yevgenii Velikhov, did not lack openness; Primakov brought up the troubles in Afghanistan, suggesting that US speakers might have avoided the topic out of politeness. The view of arms policy they brought with them reinforced the views expressed earlier by Stockholm Peace Research Institute director Walter Stuetzle that Western leaders seem not to have gained "intellectual parity" with Gorbachev.

The Soviet Union, Velikhov said, "is not interested in a less secure United States, because a less secure United States is more aggressive and the situation more tense". The old Geneva negotiating style of "trading warheads", to win a concession from the other party, is "wrong". A new approach is needed, he said, in which the goal is "better stability".

That view is not one that has been much apparent in arms negotiations. SALT II, the last major US-Soviet arms agreement, Velikhov said, was in "fundamental error". Many of his Western colleagues would agree. By placing limits on launcher categories, rather than on warhead numbers, the treaty encouraged both the United States and the Soviet Union to put highly accurate warheads on their missiles.

The resulting nuclear force is of the worst kind, capable of carrying out an overwhelming first strike against military targets with its huge number of warheads (4,000 on Soviet SS 18s and 19s, 2,650 on US Minuteman IIIs and the MXs now being deployed) while so vulnerable that launch on first warning of attack is encouraged. This force, Velikhov said, contrasts with the more stable deterrence of the submarine fleet, where communications difficulties and lesser missile accuracy give poor potential for coordinated surprise attacks, while the fleet's invulnerability ensures that attackers cannot escape retaliation.

Putting the United States and the Soviet Union back into a stable nuclear configuration will not be easy. The Soviets have far more strength tied up in massive land-

based missiles and pressure for change will be felt to fall unfairly upon them. Nevertheless, Velikhov said that the hoped-for agreement to cut nuclear forces by 50 per cent could be reached by "next spring". As cuts go deeper than that, the problems posed by Strategic Defense Initiative (SDI) would increase, he said. Any kind of deployed defence system would hit at the underlying philosophy of maintaining forces that are too small for a pre-emptive strike, but sufficiently invulnerable to ensure retaliation.

Velikhov also presented results from the collaboration between the Academy of Sciences of the USSR and the National Resource Defense Council (see *Nature* 328, 192; 1987), which took a team of US seismologists to the Soviet Union. The problem of verification of a test-ban treaty is "solved" he said. Monitoring of a non-nuclear test explosion at distances of 250 and 600 km from the Semipalatinsk test site showed that an explosion could neither be hidden in natural earthquake tremors nor decoupled from the surrounding rock.

Alun Anderson

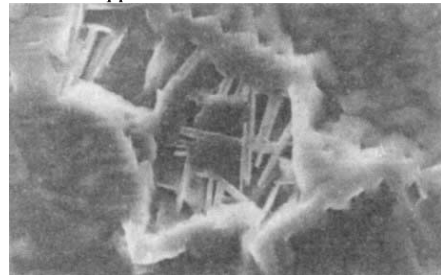
Superconductors by new melt process

Washington

A SUPERCONDUCTING yttrium-barium-copper oxide compound that retains its critical current density in a strong magnetic field has been developed at AT&T Bell Laboratories.

Using a technique called melt-textured growth, Sungho Jin, Thomas Tiefel, Richard Sherwood and Bruce van Dover created a sample that had a critical current density of 7,400 amps per cm² at zero magnetic field, and 1,000 amps per cm² in a 1-tesla magnetic field at 77K.

In the new process, powders of yttrium, barium and copper oxides in a 1:2:3 ratio are heated



An electron micrograph of the 'needle-like' structures that result from melt-textured growth. to 1,300 °C, past their melting points, then cooled rapidly. The resulting compound is then annealed. The morphology of the melt-textured growth micro-crystals is decidedly different from those formed by sintering where the rare-earth powders are kept below their melting points. The results were presented last week at the Materials Research Society meeting in Boston.

Joseph Palca

Conference acts on arms control policy education

Palo Alto

THIS week's conference at Stanford was part of the continuing effort of the Center for International Security and Arms Control to educate the public, and scientists in particular, in the issues of arms control policy. Two physicists, Wolfgang Panofsky and Sidney Drell, now respectively director-emeritus and deputy director of the Stanford Linear Accelerator Center, set up the centre fifteen years ago together with political scientist John Lewis.

All remain active in the centre's affairs, Drell and Lewis as co-directors, and have seen it grow to the largest arms control study centre in the United States. Its 70 members are much in demand as advisers.

Drell, now 61, says he "keeps getting pulled back to his physics" but retains a particular interest in running the centre's science fellowship scheme. This provides three fellowships a year to train mid-career scientists in arms control issues.

The aim is to ensure that there is a new generation of scientists, competent both scientifically and politically, who can succeed such earlier leaders as Hans Bethe, Charles Townes and Richard Garwin. Drell also runs a weekly seminar on technical issues in arms control.

The quality of scientific advice received by the administration and the responsibility of scientists to speak accurately on technical issues much exercise Drell. He regrets the lack of authoritative advice at the White House since the abolition of the President's Scientific Advisory Committee in 1973. During the present administration, Drell says, the signs are that there has been little scientific advice on defence issues. Drell notes that Nobel-prizewinning physicist John Bardeen, although then a member of the White House Science Council, was not consulted before President Ronald Reagan suddenly launched the SDI in March 1983.

But President Reagan is taking at least some advice from Stanford. Just down the road from Drell's office is the Hoover Institution and Edward Teller, who has much encouraged the president's faith in SDI. Indeed, as the arms control conference opened in Washington, Teller was in the news again with enthusiastic support for an early deployment of missile defences.

Drell backs the view that research on SDI should continue but that deployment must be restricted by the conventional interpretation of the ABM treaty. Which view is to prevail is likely to be a critical issue at this week's summit.

Alun Anderson

Changing features sighted on the biotechnology horizon

Washington

THE landscape of federal support for life sciences has a new feature. A committee on life sciences has formed under the umbrella of the Federal Coordinating Council of Science, Engineering and Technology (FCCSET), to be chaired by Beverly Berger of the White House Office of Science and Technology Policy (OSTP).

Changes in coordination of biotechnology have been expected since the charter for the Biotechnology Sciences Coordinating Committee (BSCC) expired at the beginning of last month. BSCC had no statutory authority but was nevertheless influential. BSCC and its chairman David Kingsbury, associate director of the National Science Foundation (NSF), promulgated the coordinated framework for biotechnology, an attempt to rationalize federal biotechnology regulations.

But BSCC's role and authority became murky this year. As a committee organized under FCCSET, it is subject to authorization from OSTP director William Graham, and there were signals from OSTP that changes were afoot. Complicating matters was the investigation of NSF's Kingsbury for possible conflict of interest charges over his alleged management role in a subsidiary of IGB Products, a British biotechnology holding company, while at NSF.

Under the reorganization, BSCC will continue to function. James Wyngaarden will become chairman, and NSF deputy director John H. Moore will replace

Kingsbury on the committee. BSCC will retain its old functions, and will also liaise with the new FCCSET life sciences committee. The new committee will have a broader membership than BSCC, including representatives from the Office of Management and Budget, the US Trade Representatives Office and most cabinet-level agencies. Berger has declined to discuss the committee's agenda until its first meeting.

Perhaps the most important role for the new committee will be its oversight of federal activities on the human genome.

Before coming to OSTP, Berger worked for the Department of Energy in the Office of Health and Environmental Research, the office responsible for the Energy Department's human genome activities. There has been some jealousy between federal agencies over who would be leading any federally sponsored mapping effort. The Energy Department and the National Institutes of Health, the two agencies with the largest roles thus far, have recently been working together more congenially, a contrast to some sharpish words spoken earlier in the year.

OSTP's interest in coordinating federal research on the human genome is shared by some in Congress. This week, Senators Kennedy, Chiles and Domenici were expected to introduce legislation that would establish a committee to oversee federal genome activities. The new year may bring even more interested parties.

Joseph Palca

Strawberry plants attacked in Californian field tests

San Francisco

IN the latest incident in California's battle between environmental groups and biotechnology, sabotage has delayed a test of genetically engineered frost-preventing bacteria by the Oakland-based company Advanced Genetic Sciences (AGS).

On 30 November, just two days before the test was to begin, vandals slipped past guards, scaled a fence and spread rock salt on the test site, a strawberry field east of San Francisco. The radical environmental group Earth First!, which advocates sabotage, claimed responsibility.

So far, all three California field tests of genetically engineered bacteria — two by AGS and one by the University of California at Berkeley — have been damaged by vandals.

Although the environmental groups have not succeeded in stopping the field tests, they have in some cases reduced

their scientific value, and have cost both AGS and the University of California millions of dollars in delays and court battles.

Trevor Suslow, director of product research for AGS, said the rock salt was removed from the field and the plants seem not to be damaged. But, as the vandals claimed to have sprayed a slow-acting herbicide as well, AGS will wait a few days before proceeding with the experiment.

The first AGS field test last April was declared a success after no genetically engineered bacteria were found outside the test site and laboratory tests showed the bacteria to confer several degrees of frost protection on the plants. But the vandals had removed many of the blossoms needed for study, and court delays had pushed the test so late into the spring that the plants were not exposed to a

Orphaned embryos to be implanted

Sydney

AFTER being frozen for six years and in legal limbo for four, two orphaned embryos are to be given a chance to live and breathe.

The embryo's parents, Elsa and Mario Rios, were killed in an aeroplane crash in Chile in April 1983, leaving an \$8 million estate. The Rios came to Australia from California in 1980 to take part in the *in vitro* fertilization programme of Melbourne's Queen Victoria Hospital which had no equal in the United States at the time.

Court rulings on both continents were needed to get the embryos out of the deep freeze. In May 1985, a California Superior Court decided not to appoint legal guardians for the embryos and declared that they were not legal heirs to the Rios estate, nor were they its property.

In Victoria, action had to wait for the proclamation of legislation giving the Minister for Health, Mr White, the power to decide the fate of embryos without parents.

The embryos will be implanted in a prospective mother who cannot produce eggs of her own and whose husband cannot produce sperm. Because of the less sophisticated techniques used when they were frozen in November 1981, the chances of either of the embryos surviving the rigours of thawing and implantation are thought to be only 1–2 per cent.

According to White, if the embryo implantation is a success, the children who are born would not be considered relatives of surviving members of the Rios family. He said that the California decision will serve as a precedent in Australia and future orphaned embryos will have to be considered case by case.

In the future, prospective parents depositing prospective children in storage in Victoria will have to sign a declaration setting out what is to become of them should the parents die. Charles Morgan

natural frost. The current test, expected to last for 4 months, should expose the plants to frost in the field.

Suslow said that AGS needs several more field tests before its frost-preventing bacteria will be ready for market. He said the company will not give up Californian field tests, despite the harassment. But as the Organization for Economic Co-operation and Development irons out its regulations for such tests, the company may look abroad for countries more sympathetic to environmental release experiments. Several countries, including Italy, have shown an interest in tempting US biotechnology companies to set up shop outside the United States.

Marcia Barinaga

Byelorussian intrigue takes new turn

London

THE former president of the Byelorussian Academy of Sciences, Mikolai Barysevich, who was replaced last March, exerted "inadequate control" over the institutes of the academy, according to a report in *Sotsialisticheskaya Industriya*. In particular, the paper said, this lack of control had led to an "abnormal moral and psychological climate" in the academy's Nuclear Power Institute. The director of the institute, V. Niescerau, was relieved of his post in July; a committee of enquiry, however, put much of the blame on the leadership of the academy, and, in particular Barysevich.

What precisely went wrong at the Nuclear Energy Institute is unclear. A handbook on the "scientific potential" of Byelorussia, published earlier this year, speaks well of its work on gas-cooled fast-breeder reactors and applications of gamma-radiation in the production sector. Nor was the replacement of the ageing Barysevich by 48-year-old Uladzimir Platonau viewed, at first, as anything more than the implementation of Mr Gorbachev's policy of "rejuvenating" all sectors of Soviet life. Barysevich was a physicist of some repute, who in 1980 received a Lenin Prize for his work on lasers, and initial reports had suggested simply that he was going into honourable retirement.

There is, however, one piece of evidence which may suggest that several months before his replacement, Barysevich was aware of criticisms of slackness, and tried to counter them before they became official. In autumn 1986, he made the allegation, expressed in the weekly *Liternaya Gazeta*, that Professor Hollis B. Chen of the University of Ohio had plagiarized the work of the Byelorussian physicist Fiodar Fiodarau. (Since Barysevich's own scientific reputation was assured, any pending criticisms would refer, rather, to his role as leader of the academy — and his defence of Fiodarau could be cited as evidence of his concern for the interests of his academicians.) The controversy dragged on for several months, becoming if anything stronger after a commission of enquiry set up by Ohio University pronounced Chen not guilty of deliberate plagiarism. Then, almost immediately after Barysevich was replaced as president of the Byelorussian Academy of Sciences, the campaign stopped. This may be simply coincidence. But it is noteworthy that Fiodarau, the 'victim' of the alleged plagiarism, who never, incidentally, made any public charges on his own behalf, is now in friendly correspondence with Ohio University, including the members of the commission which cleared Chen. Vera Rich

First steps towards preserving the Earth's ozone layer

Washington

To maintain the international harmony achieved in the signing of the Montreal Protocol to reduce atmospheric ozone depletion, the US Environmental Protection Agency (EPA) has decided to cut back production of chlorofluorocarbons (CFCs) and halons precisely in line with the minimum demands of the protocol, but no further.

CFC production will first be frozen at 1986 levels, then reduced by 20 per cent in 1993 and finally by 50 per cent in 1998. Halons, which are not yet thought to be significantly active in ozone destruction, will be locked at 1986 production levels after 1992. These reductions are supposed to ensure that global ozone levels remain unchanged and stable in the long term.

The proposed rules have been widely

criticized by a number of environmental groups, which claim that EPA, under pressure from business interests and the Reagan administration, is backsliding. Previous EPA studies showed that an 85 per cent cut would be needed simply to keep atmospheric CFC concentrations from increasing.

Although the Montreal agreement specifically allows participating nations to take more stringent action, EPA administrator Lee Thomas says that several countries were not at the outset convinced that any cuts were needed, and he regards it as important to "keep faith" with the compromise. But it is also true that industry has consistently lobbied hard against unilateral action on the grounds that it would damage the United States in the world market.

David Lindley

Britain's synchrotron partners reject 10 per cent solution

London

FRANCE and West Germany are blocking Britain's membership of the European Synchrotron Radiation Facility (ESRF), complaining that a subscription fee equivalent to 10 per cent of the project's total cost is too little. At last week's meeting of ESRF's provisional council in Paris, it was made clear that Britain's offer was not acceptable. The governments of the three countries must now reach an agreement before the end of this year.

Disappointed British delegates had lobbied strenuously for a share of the domestic science budget. A few weeks earlier, the Science and Engineering Research Council (SERC) had announced that it could afford no money towards ESRF membership. But a week before the Paris meeting, the Advisory Board for the Research Councils, which advises the government on how the science budget should be distributed, allocated extra money to SERC, sufficient for a 7 per cent subscription to ESRF, with SERC itself finding the remaining 3 per cent.

Although the British contingent was aware that the other member states would be looking to Britain to shoulder nearer 14 per cent of the costs of the 11-year, £350-million project, it hoped that any objections would be cleared up on the day.

The memorandum of understanding between the member states that permitted a £3-million foundation study to begin two years ago expires at the end of this year. For the construction phase to start, an international convention is needed, which is likely to take up to six months to com-

plete (certain member states require parliamentary approval). In the meantime, an interim intergovernmental protocol has been agreed that will allow work to begin on time, nominally on 1 January next year.

Provisional subscription levels (apart from Britain's) were agreed last week, with France, the host nation for the Grenoble-based venture, agreeing to contribute up to 38 per cent and Germany up to 28 per cent (depending on Britain's eventual contribution; in either case not less than 35 per cent and 25 per cent respectively), Italy 15 per cent, and 4 per cent each (the minimum allowable contribution) from Switzerland, Spain and a Nordic consortium comprising Norway, Sweden, Denmark and Finland.

Despite last week's setback, British synchrotron users remain hopeful that ministers will reach an agreement within the next fortnight that will secure Britain's membership of the consortium. One possible compromise would be to extend the principle of *juste retour* (where participants in international ventures are awarded staffing and building contracts in proportion to their contributions) to beam-time, with member states allocating their share of the time to their own scientists. Britain's synchrotron community, which would probably stand to lose by such a system, argues that abandonment of the present arrangement, where projects are assessed by their scientific merit by international peer review, would diminish the quality of the science.

Simon Hadlington

Boycott of South Africa

SIR—The article on the academic boycott of South Africa (*Nature* 327, 259; 1987) contained various views, but nevertheless evidenced the value of even limited insight into South Africa's socio-scientific problems; but as a South African, I find the subsequent letters by Wilson, Stein and Wallis (*Nature* 328, 288 & 374; 1987) shortsighted and misinformed.

Wilson's letter is filled with vague assumptions and innuendos, for example "... some white South African scientists ... secretly welcome the boycott ...". Nobody I know supports this nonsense, and I challenge Wilson to prove this statement. Wilson's likening of the South African scientific and social scene to a war shows a total lack of insight into local conditions. The international media's lust for portraying necklace murders should provide sufficient evidence of who is doing what to whom. While African countries such as Ethiopia, Chad, Mozambique and Angola, where wars are raging, experience large dislocation of people and endless refugee problems, South Africa experiences an influx of foreign Africans wanting security and employment. How can Wilson say "... 17 million black South Africans are denied the possibility of a scientific education and career" when there are more South African black children in primary schools than there are white people in this country? Wilson refers to "... the people seeking to establish a non-racial democracy ...". The track record of most previous colonies show that the number of democracies that emerged after the independence wave hit Africa in the 1960s can be counted on one hand. I would sincerely like to know whether Wilson has equally simplistic views on the situation in Northern Ireland.

Both Wilson and Stein refer to the possibility of scientists resettling during or after the so-called "change-over". After the string of broken international promises to whites in pre-independent Zimbabwe, the last thing many South African scientists will do is to accept promises of resettlement. In fact, we have come to see little good in "international" promises of any kind.

It is appropriate briefly to dwell on the possible future structure of South African science. I disagree strongly with the view that local science would be "dead" if isolated from the international scientific community. South Africa has so much going for it that the Jonahs of this world are bound to be disappointed.

First, I recall similar statements of imminent doom when South Africa left the British Commonwealth, or was barred from the United Nations plenary sessions, or when military or limited economic

sanctions were applied, or even when sporting ties were severed. None of the predictions materialized, simply because sanctions do not work. I believe that South Africans are faced with a similar challenge in the scientific field and that the international community can similarly prepare itself for disappointment. I also believe that communication (whether scientific or otherwise) has benefits to all concerned. Foreign scientists visiting this country normally leave with a new perspective of the problems facing us, and they succeed in creating a similarly widening view among the local fraternity. Understanding is already a significant step on the road to solutions. To challenge the writers' seemingly naive approach, I would like to ask whether you would change under threats of boycotts and isolation?

Second, the South African environment (unlike that in many industrialized and developed countries) contains many fields that have been virtually untouched by local science. A few examples will suffice: South Africa has largely unexplored deep-ocean features off its coasts such as the Agulhas current, eddies and fronts. It has an important fisheries zone in the Benguela region, much of which is still unknown. There is no local expertise to evaluate and prepare for predicted sea-level rise of the next century. We have only a rudimentary understanding of the role oceans play in local and global climate and weather. Many problems surround the occurrence of the giant waves in the Agulhas current that have caused considerable financial and other losses to international shipping. We have to grapple with an increasing demand on the coastal environment in terms of recreation and pollution. These are all features that can be or are being tackled with little international involvement, but in which there can be an extremely fruitful collaboration.

Obviously, the thoughts expressed here are my personal views. But I do challenge Wilson, Stein and Wallis to name one other country where problems (scientific, social and other) such as those in South Africa have been solved successfully. Until then, South African science will be well advised to concentrate on its own affairs, rather than become yet another misconstrued and discarded experiment on the road of international pseudoscience.

MARTEN LUTHER GRÜNDLINGH

24 Kaneel Crescent,
Stellenbosch 7600,
South Africa

SIR—I would like to make the following points about the recent correspondence on South Africa.

(1) The concept of a scientific boycott for political reasons is dangerous, because

anybody could easily become an 'outlaw'. It generates manicheist 'holier than thou' reactions. If it is acceptable for South Africa and apartheid, why not boycott the scientists of the many countries where apartheid is applied to the whole population, where nobody votes and unions and strikes are unheard of, where machine guns at the border are turned inwards to prevent people from voting with their feet and leaving?

(2) Attempts to strike at individuals, for example by boycott, on the basis of nationality, race or belief smack of precisely the attitude that generates apartheid.

(3) More fundamentally, scientists do not represent their government or their country; they represent themselves — individual scholars in search of the truth and dedicated to sharing their feelings.

Let us not make science the object of political football that sport has become.

J.E. DUMONT

Chemin du Chêne-aux-Renards, 32,
1328 Ohain, Belgium

Doom and gloom

SIR—Efraim Racker¹ enlivens the scene by arguing with Chargaff in a collision of optimism versus pessimism, and ends by joining Chargaff's familiar lament for what's wrong with the world. Racker does not comment, however, on Chargaff's ghoulish prophecy of "a gigantic slaughter-house, a molecular Auschwitz, in which valuable enzymes, hormones and so on will be extracted instead of gold teeth"². Chargaff has worked himself into a fine lather, but, it seems, over nothing. Surely he must have heard that recombinant DNA technology, his *petit-enfant terrible*, supplies us with enzymes and hormones, thus doing away with the need to extract them from animal tissues? But perhaps he has not: he usually appears to reject his grandchild, the double helix, for its misbehaviour in escaping from the quiet serenity of his biochemical laboratory, first into the hands of its foster parents ("two pitchmen in search of a helix"³), next into a "publicity carnival"³ and now, into the market-place.

Elsewhere in his Commentary, Chargaff might have acknowledged (but didn't) that his description of "mass production and industrial exploitation of human embryos" was developed in much more detail by Aldous Huxley⁴, in a book first published in 1935.

THOMAS H. JUKES

Department of Biophysics
and Medical Physics,
University of California, Berkeley,
Oakland, California 94608, USA

1. Racker, E. *Nature* 329, 100 (1987).

2. Chargaff, E. *Nature* 327, 199–200 (1987).

3. Chargaff, E. *Nature* 248, 776–779 (1974).

4. Huxley, A. *Brave New World* (Harper, New York, 1946).

Academic vacuum on arms control

This week's hoop-la in Washington should not conceal the need for more rigour, but also more intellectual adventurousness, in providing politicians with a means of making progress towards arms control.

THE successful negotiation of the agreement to get rid of Euromissiles, signed in Washington this week, has stimulated the appearance in international newspapers of advertisements in the names of public figures, most of whom appear to be retired generals, pleading (in the *International Herald Tribune* for 7 December) STOP THE INF TREATY!. The advertisement, evidently concerned to influence the debate in the US Senate to begin after the Christmas recess and attributed to an address in West Germany "c/o the Schiller Institute" at Hannover, makes one telling point: the day (last Tuesday) on which Mr Gorbachev and President Reagan signed the treaty was the anniversary of the Japanese attack on Pearl Harbour, in 1942 a US base in the Philippines.

Otherwise, there would be few whose attention would be caught by what the retired generals have to say. Indeed, the idiom of the advertisement is so redolent of ancient language (such as "Moscow" for "Soviet government") that it reads as if it were an almost mediaeval document. Surely, many readers of the advertisement will have asked, this is the language of the past? Before supposing that asking the question presupposes assent, people might stop to ask what is the present scholarly basis of arms control, and whether it is sufficient.

It is a curious business. After a decade of cold war (the 1950s) there was a remarkable flowering of interest in the machinery of arms control. To some extent, this was determined by the extent to which diplomats with inclinations (or instructions) to negotiate test-bans found themselves ignorant of the principles by which compliance with such agreements might be verified; they naturally turned to the people who knew about the construction of weapons and about seismology. By 1960, it was well-established that technical people had an important contribution to make to international diplomacy.

From that point on, but for about a decade only, academic initiatives directed towards the techniques of arms control flourished remarkably. In the United States, the running was made by the President's Scientific Advisory Council (PSAC), largely consisting of people whose background and preoccupations equipped them to comment on strategic issues. Private foundations such as Carnegie and Fords quickly followed suit. In Britain, organizations such as the Institute

of Strategic Studies (now the International Institute for Strategic Studies), already in being, flourished as general interest grew in the prospect that the cause of arms control might be advanced by intellectual activity of a familiar kind.

The 1970s, unfortunately, showed that conceit to be an illusion. Professor Sydney Drell (see page 511) is right to regret the abolition by President Richard M. Nixon of his PSAC, but the grounds for his regret may not properly be those stated. Advice on general matters can always, if unwelcome, be ignored. PSAC's public (but not presidential) service, during the great antiballistic missile debate raging on the eve of its abolition, was to engage the interests of the US academic community in an issue that would otherwise have seemed exclusively a matter for the military.

It is no great surprise that, with PSAC's abolition, the interest in academic studies relevant to arms control has substantially declined. Even the novel problems evoked by the US president's announcement in 1983 of his Strategic Defense Initiative (SDI) have not recreated the excitement of earlier decades. Part of the explanation is that the military have not been eager to provide funds for studies whose outcomes might be unfavourable, but there is more than that to be said.

Opinions are bound to differ, but the academic arms-control enterprise has become unsatisfying to its practitioners. That is at least part of the explanation why so much of the still considerable torrent of published work in the field appears stilted. People embark on theoretical schemes for the more accurate identification of underground explosions from earthquakes, or for the calculation of the numbers of satellites required to keep a missile launching point under surveillance, with all the enthusiasm of people who have designed the skeleton of a novel computer program and who must now buckle down to the task of writing all that beastly code. While embarking on that tedious task, moreover, they know that it must already have been done (and promptly classified) by people working for the military.

This is another way of saying that, public service though it may be, the business of giving advice to the military (or, more publicly, to their electors) is unexciting. The chance of making a discovery is small. The likelihood that the outcome of an intricate piece of work will

be indecisive is great. And the record shows, in any case, that policy-makers will not take much notice of it unless there are powerful advocates of its importance.

That is a sufficient explanation of the decline, since the early 1970s, of the contemporary interest in strategic studies bearing on arms control. But it is not the whole explanation. In the fashionable language, the field of study has become interdisciplinary to an unexpected degree. So much should be apparent from the verification of the treaty on intermediate nuclear missiles signed this week in Washington, which includes provisions for allowing each side to request from the other an inspection of some factory or missile silo. In the idiom of the 1960s, that would have been a simple matter of gaming theory; Herman Kahn would have had a computer simulation of the problem in a twinkling. The snag is that the act of asking for an inspection will inevitably, under the treaty, be a political act whose consequences will not be easily calculable. Can the calculators of strategic stability avoid straying into political science?

It is unlikely that new developments in mathematics will bring the subject back to simple science. To be sure, the past few years have seen several attempts to relate the instability of military situations to catastrophe theory or, more recently, to the chaos that may arise from simple non-linear equations. The results of calculations along these lines are usually suggestive of the real world, but are often also demonstrations that it is likely always to be impossible to gather empirical data accurate enough to provide more than a qualitative basis for conclusions about the real world of the strategic balance.

That is why Professor Drell may be mistaken in his belief that his Stanford centre exists to replace the generation of natural scientists who have given valiant public service in the cause of arms control during the past three decades. It could be that circumstances have changed too much for similar contributions to be as useful in the years ahead. It may even be that the arms control process itself, the process of writing treaties, has reached the limits of complexity capable of comprehension by those elected to sign them. If that is so, the academic community would not be absolved from responsibility, but the opposite. Finding new ways of tackling old problems may, indeed, be the most urgent need.

John Maddox

Evolution

Darwinism stays unpunctured

John Maynard Smith

OVER the years, palaeontologists have tended to distrust the account of the mechanisms of evolution given by population geneticists. The most recent challenge was the theory of punctuated equilibria¹. This asserted that the fossil record reveals that evolutionary changes are not gradual and continuous, but that long periods in which little or no change occurs (stasis) are interrupted by rather sudden changes (punctuation). It is further claimed that the sudden changes occur at times of lineage splitting (speciation), and it was at first argued that they occurred in isolated peripheral populations. Finally, in some more extreme statements of the theory^{2,3}, it is argued that the changes are not brought about by natural selection within populations, and that they are random relative to the overall trends of long-term evolution. But the latest contribution to the debate, from P.R. Sheldon on page 561 of this issue⁴, suggests that punctuation is not a universal pattern.

If punctuational changes are random, then the main features of evolution are not the summed consequences of changes caused by selection within populations, of the kind that can be studied today: macroevolution is decoupled from microevolution. How, then, is the adaptation of existing organisms to their ways of life to be explained? One response⁵ has been to deny that adaptation is as pervasive as it seemed to Darwin, and has seemed to most naturalists since. But adaptation cannot be wholly denied. It has therefore been suggested that it arose by 'species selection'. This is a process exactly analogous to selection within populations, in which individuals are replaced by species, birth by speciation, death by extinction, and mutation by the non-adaptive punctuational changes that occur when new species arise.

Geneticists, needless to say, have been unimpressed by the latter part of this argument. They have pointed out that species selection is quantitatively inadequate to account for the degree of adaptation observed, and that the claimed absence of intermediates between species is disproved by the facts of geographical distribution: every degree of difference can be observed between isolated populations from almost no difference up to differences as great as that separating good sympatric species. They have also noted that, in the two studies most often quoted in support of punctuational theory (Eldredge⁶ on trilobites, and Williamson⁷ on the molluscs of Lake Turkana),

populations intermediate between the earlier and later 'species' were found. These populations could not have been small, and it is therefore hard to see how the changes that took place in them could have been caused by anything except within-population selection (unless, as has been suggested for Williamson's molluscs, the changes were not genetic at all).

It would be quite possible, however, to accept the claim that the typical pattern of change is one of long periods of stasis interrupted by brief periods of rapid change, without also accepting the ideas of non-adaptive change, species selection,

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Cnemidopyge, Ordovician trilobite from central Wales. (Courtesy of P.R. Sheldon.)

and the uncoupling of macro- and microevolution. This is a question for palaeontologists to settle.

Sheldon in this issue⁴ reports his study of eight genera of Ordovician trilobites, over about 3 million years, from central Wales. The classification of the group into species is largely based on the number of pygidial ribs — features of the exoskeleton (see figure). Sheldon examined about 15,000 specimens, from seven sections and 400 sampling sites, and was able to count the ribs of about 3,500 of them. In all eight genera, the number of ribs increased during the period, although there were changes of rate and several temporary reversals of direction.

The overall difference between the earliest and latest populations studied by Sheldon is such that they had, in all cases, been placed in different species, and in one case in different genera. But in no case is it possible to recognize a punctuational event marking the transition from an earlier to a later form. Most populations are intermediate between the

previously described species, and some are sufficiently variable to include specimens that, according to the previous criteria for classification, would have been placed in different species. However, these variable populations are probably not to be interpreted as mixtures of two species, because most are unimodal in rib count, and the few that are not can best be interpreted as reflecting a relatively rapid shift in mode. In fact, Sheldon thinks that no genus was represented by more than one species at any one place and time, and there is no sign of lineage splitting in any of the genera.

These results have implications for the application of linnaean nomenclature to fossils. Communication requires that we give names to fossils, and convenience suggests that they had better be linnaean names. Before Sheldon's study, these trilobites could successfully be assigned to linnaean species, and, as he remarks, this could have been interpreted as evidence for punctuation and stasis. His new, more detailed study shows that this interpretation is unjustified. The conclusion is that, no matter how we decide to name fossils, we should make statements about rates and patterns of evolution only on the basis of a statistical analysis of populations, and not on the presence or absence of named species or genera. I do not think that the attempt to explain morphological evolution by species selection can survive in the face of results of this kind. But there never was much sense in the idea anyway.

Stasis, however, is a different matter. As David Wake pointed out during an earlier debate on this topic, the existence of sibling species demonstrates the occurrence (but not, of course, the universality) of stasis. For example, the fruitflies *Drosophila melanogaster* and *D. simulans* are hard to distinguish morphologically: the only plausible explanation is that neither has changed appreciably in morphology since their divergence from a common ancestor several million years ago. Geneticists have tended to explain such stasis by normalizing selection for an unchanging optimum, and palaeontologists by developmental constraints: no doubt we shall continue to argue about their relative importance. But my own view, which will not be universally shared, is that we can forget about new paradigms and the death of neodarwinism. □

1. Eldredge, N. & Gould, S.J. in *Models in Paleobiology* (ed. Schopf, T.S.M.) 82–115 (Freeman, San Francisco, 1972).
2. Gould, S.J. *Paleobiology* 6, 119–130 (1980).
3. Stanley, S.M. *Macroevolution* (Freeman, San Francisco, 1979).
4. Sheldon, P.R. *Nature* 330, 561–563 (1987).
5. Gould, S.J. & Lewontin, R.C. *Proc. R. Soc. B* 205, 581–598 (1979).
6. Eldredge, N. *Bull. Am. Mus. Nat. Hist.* 47, 45–114 (1972).
7. Williamson, P.G. *Nature* 293, 437–443 (1981).

John Maynard Smith is in the School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK.

G proteins and cAMP

Discovery of a new oncogene in pituitary tumours?

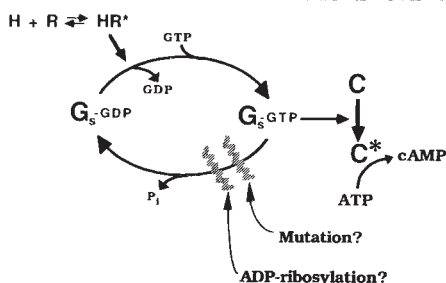
Henry R. Bourne

ANY gene can aspire to become an oncogene, providing that its protein product generates a signal that tells a cell to proliferate. Many trophic hormones use protein machinery of the cyclic (c)AMP signalling pathway to promote growth of their target cells. Why, then, have attempts to establish cAMP as a mediator of oncogene action so often come to grief^{1,2}? Part of the reason may be simply that previous investigators looked at the wrong set of tumours. That speculation is triggered by the discovery of hyperactive cAMP synthesis in a subset of human pituitary tumours, described by Vallar *et al.* on page 566 of this issue². These investigators report increased activity of G_s , the stimulatory regulator of adenylyl cyclase^{3,4}, in pituitary adenomas that secrete growth hormone (GH). The enhanced G_s activity probably accounts for increased GH secretion by the tumours and may even have triggered their neoplastic growth. Indeed, it may turn out that the α_s -polypeptide chain (α_s) of G_s in these tumours is the biochemical target or translation product of a new oncogene.

From measurements of GH secretion and adenylyl cyclase activity, Vallar *et al.*² divide more than 50 human pituitary tumours into two groups. In group 1, as in normal rat pituitary cells, GH releasing hormone (GHRH) stimulates GH secretion and elevates both cellular cAMP and the adenylyl cyclase activity of broken-cell preparations. By contrast, tumours of group 2 show striking basal elevations of GH secretion, cAMP content and Mg^{2+} -dependent adenylyl cyclase activity, all three to levels considerably higher than those of group 1 cells stimulated by GHRH. Synthesis of cAMP in group 2 cells seems to be constitutively 'turned on' at quite a high level, and it fails to respond to further stimulation by GHRH. Other stimulatory agents that act via G_s — including a hydrolysis-resistant GTP analogue, GTP itself, fluoride ion and cholera toxin — stimulate adenylyl cyclase in group 1 but slightly inhibit the high enzyme activity of group 2 tumours.

The constitutive turn-on of cAMP synthesis in group 2 tumours could result from alterations in any one of the three well-characterized components of hormone-sensitive adenylyl cyclase activity^{3,4}: hormone receptors (R), the adenylyl cyclase catalytic unit (C), or G_s , the regulatory protein that couples R and C. Like other members of the G-protein family, G_s normally oscillates between an inactive

GDP-bound form and an active GTP-bound form that can associate with and stimulate the effector — in this case, C (see figure). Hormone-receptor (HR) complexes activate G_s by promoting replacement of GDP by GTP in the guanine nucleotide binding site of α_s . G_{s-GTP} then stimulates C until the intrinsic GTPase activity of α_s hydrolyses the bound GTP, thereby inactivating the protein and preparing it for activation by another



Activation cycle used by G_s to mediate stimulation of adenylyl cyclase (C) by hormone-receptor (HR) complexes. Wavy bars indicate defects that might account for the neoplastic phenotype and the constitutively elevated cAMP of type 2 pituitary tumours (see text).

HR complex. Two experiments implicate G_s as the altered component in group 2 tumours. In the first, Vallar *et al.*² assess G_s activity in detergent extracts of tumour membranes by biochemical complementation of the α_s -deficient membranes of S49 lymphoma cyc^- cells. Extracts of group 1 membranes allow the adenylyl cyclase of cyc^- to respond to stimulation by NaF; in contrast, a mixture of group 2 extracts with cyc^- reproduces the pattern of activities seen with the group 2 membranes themselves, that is, greatly elevated basal activity slightly inhibited by GTP and NaF. The simplest explanation of this experiment is that the group 2 extracts contain constitutively activated G_s molecules. Because C in the complementation protocol comes from cyc^- rather than from the tumour extracts, the experiment excludes C as the primary site of abnormality in group 2. It does not, however, absolutely exclude the possibility that group 2 membranes contain a constitutively activated R component (R capable of activating G_s in the absence of stimulating hormone); this is less likely, in part because it requires simultaneous incorporation into cyc^- of both exogenous R and exogenous G_s , a technical feat without precedent in the cyc^- reconstitution system.

The second experiment also points to

G_s . Vallar *et al.*² radiolabelled G_s in tumour membranes by taking advantage of the specific ability of cholera toxin to catalyse transfer of ADP-ribose from radioactive NAD⁺ to α_s . In comparison to membranes from group 1, radiolabelling is strikingly reduced in those of group 2 tumours. This observation is difficult to explain on the basis of changes in either R or C activities, but could easily result from a change in G_s (see below).

Here a qualification is in order: the key experiments pointing to G_s involve a very few tumours (two from each group for the biochemical complementation of cyc^- membranes, and only one from each for radiolabelling by cholera toxin). This is probably because GH-secreting pituitary tumours are hard to come by and because their small size provides too little tissue for complete biochemical study. Consequently, Vallar *et al.* must extrapolate from a few key determinations to infer that altered G_s is responsible for the elevated cAMP, adenylyl cyclase and GH secretion they observe in the much larger number of tumours included in group 2. Despite this qualification, the postulated alteration of G_s fits the phenotype of group 2 tumours quite neatly — so much so that one is inclined to accept it, at least provisionally, until more complete results become available.

What, then, is wrong with G_s in group 2 tumours? The most interesting possibilities involve either covalent modification or an activating mutation. Both have well-established precedents in GTP-binding proteins, and both are easily testable. As an example of the first possibility, α_s in group 2 could be modified by an endogenous ADP-ribosyltransferase similar to cholera toxin. Affected tumours could overexpress the modifying enzyme, or they could lack an enzyme that reverses the modification. The postulated modification would stabilize G_s in its GTP-bound active state and the modified G_s would behave like G_s that has been ADP-ribosylated by cholera toxin^{3,4} — as is the case in group 2 tumours, the modified α_s chain could not be radiolabelled by toxin and it would stimulate adenylyl cyclase in the absence of other stimulating agents. One objection to this notion arises from the failure of persistent experimental efforts⁵ to identify an endogenous enzyme that can activate G_s by catalysing its ADP-ribosylation. Nonetheless, it should be a simple matter to determine whether extracts of group 2 (but not group 1) tumours can activate G_s enzymatically, either by catalysing ADP-ribosylation or in some other fashion.

The second possibility is a somatic mutation in group 2 tumours that activates G_s directly. Precedents for this possibility abound. Of the G_s subunits, α_s would be the most likely site for an activating mutation, by analogy with mutations that

activate the related *ras* proteins. The *M*, 21,000 *ras* proteins bind and hydrolyse GTP in a presumptive pocket similar to the GTP-binding domains of several other proteins, including G protein α -chains⁶⁻⁸. Mutational replacement of any one of several residues in the nucleotide-binding pocket of the normal cellular *ras* proteins (see ref. 9 for review) produces proteins with reduced intrinsic capacity for hydrolysing GTP and markedly diminished sensitivity to a GTPase-activating regulatory protein¹⁰. Expression of these activated *ras* proteins causes malignant transformation of cells *in vitro* and contributes to oncogenesis in animals, including man⁹.

Like α , the closely related RAS proteins of the yeast *Saccharomyces cerevisiae* stimulate adenyl cyclase in a GTP-dependent fashion. A mutation that reduces the intrinsic GTPase activity of RAS in yeast¹¹ produces a phenotype remarkably similar to that of group 2 pituitary tumours: as in the tumours, adenyl cyclase in the mutant yeast cells is high and resists stimulation by agents (GTP analogues) that activate adenyl cyclase in wild-type yeast. Furthermore, the cAMP elevation caused by the RAS mutation leads to uncontrolled proliferation of yeast cells.

How can a postulated activating mutation in α account for the failure of cholera toxin to radiolabel the polypeptide in group 2 tumours? Here we get no help from the analogy with *ras* proteins, which are not subject to modification by cholera toxin. But we can speculate that the α -chain arginine residue that is modified by cholera toxin^{3,4} is not accessible for ADP-ribosylation in the mutant α , either because of an unfavourable conformation or because the mutation has replaced the arginine residue itself (perhaps with a negatively charged amino acid whose side chain somehow mimics the activating effect exerted by the negative charges of ADP-ribose).

Although speculative, this explanation also can be tested: α sequences in complementary DNA libraries from pituitary tumours could identify an α mutation in group 2; expression of the mutant polypeptide in *cyc* cells could then determine whether the mutation constitutively activates G_i. If so, the mutant α -gene would join a rapidly expanding list of oncogenes, each of which encodes an altered form (or expresses an inappropriate amount) of a normal regulatory protein, such as a growth factor, receptor tyrosine kinase, *ras* protein or cytoplasmic kinase. Whereas the biochemical pathways initiated by these oncogenes remain shrouded in mystery, the cytoplasmic 'second messenger' of the postulated α -oncogene is already well established.

In different types of normal cells, cAMP can stimulate, inhibit or have no effect on proliferation. What about the

pituitary cells that became group 2 tumours? The cAMP does stimulate GH secretion in pituitary cells and it can also promote their growth and proliferation¹², although GH-secreting pituitary cells multiply fairly slowly, even in tumours. Thus, normal α does promote growth of the appropriate class of pituitary cells, and its gene is therefore entitled to convert into an oncogene that causes pituitary tumours. Other targets of trophic hormones that use cAMP as a second messenger include the thyroid gland, adrenal cortex and gonads. It is easy to imagine that tumours of these target cells could result from activating mutations in α , or in other elements of the cAMP signalling pathway. □

1. Gottesman, M.M. & Fleischmann, R.D. *Cancer Surv.* **5**, 291-308 (1986).
2. Vallar, L., Spada, A. & Giannattasio, G. *Nature* **330**, 566-568 (1987).
3. Stryer, L. & Bourne, H.R. *A. Rev. Cell Biol.* **2**, 391-419 (1986).
4. Gilman, A.G. *A. Rev. Biochem.* **56**, 615-649 (1987).
5. Moss, J. *Clin. Res.* **35**, 451-458 (1987).
6. Masters, S.B., Stroud, R.M. & Bourne, H.R. *Protein Engng* **1**, 47-54 (1986).
7. Jurnak, F. *Science* **230**, 32-36 (1985).
8. La Cour, T.F.M. *et al.* *EMBO J.* **4**, 2385-2388 (1985).
9. Bishop, J.M. & Varmus, H.E. in *Molecular Biology of Tumor Viruses* (eds Weiss, R., Teich, N., Varmus, H. & Coffin, J.) 249-356 (Cold Spring Harbor, New York, 1985).
10. Trahey, M. & McCormick, F. *Science* **238**, 542-545 (1987).
11. Toda, T. *et al.* *Cell* **40**, 27-36 (1985).
12. Billestrup, N., Swanson, L.W. & Vale, W. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6854-6857 (1986).

Henry R. Bourne is in the Department of Pharmacology, School of Medicine, University of California, San Francisco, California 94143, USA.

Comets

Encounters of the second kind

Paul D. Feldman

THEORIES of the effects of comet showers on the Earth have been in vogue for the past few years following the suggestion that periodic variations in the geological record of mass extinctions¹, including that of the dinosaurs, could be accounted for by regular disturbances of the Oort cloud, or comet reservoir, causing massive influxes of comets into the inner Solar System^{2,3}. That subject, widely debated in *Nature* and elsewhere, appears now to be quiescent in the absence of new data. More recently, a second controversy has erupted, involving showers of significantly smaller bodies, said to continue in the present and hence to be amenable to experimental verification. On page 548 of this issue⁴, Donahue, Gombosi and Sandel offer positive evidence in a debate that, until now, has largely been confined to *Geophysical Research Letters*.

The controversy began in April 1986, when *Geophysical Research Letters* published the report and interpretation of Frank, Sigwarth and Craven^{5,6} claiming that the Earth is bombarded by 12-metre comet-like objects at the astounding rate of 10 per minute over the entire globe. The term 'comet-like object' (CLO) is preferable in this context to the usual terms 'cometoid' and 'cometesimal', as the latter imply that these objects are the building blocks of observed comets. In fact, both Frank *et al.* and Donahue *et al.* use the term only to represent extraterrestrial objects with a large water content and that originate outside the inner Solar System.

Frank *et al.* wished to explain the dark holes in the images they obtained from the Dynamics Explorer 1 satellite, of the ultraviolet dayglow emission from oxygen atoms in the upper atmosphere. They argued that the emission was absorbed by

water deposited by their proposed CLOs high in the thermosphere. Not only were their CLOs small, but also to account for the fact that they had not been seen, the mantle was suggested to be extremely dark with low porosity, so that the solar wind did not become loaded with hydrogen and oxygen ions. They also suggested, in velikovskian fashion, that these unseen objects could explain several puzzling phenomena in the Solar System.

These papers provoked a storm of comments arguing that such a high flux of small objects would produce effects discernible in measurements of atmospheric composition, in lunar cratering records, in lunar seismic data taken on the Apollo missions, or in deep-sea sediments. Others suggested that the holes were simply statistical fluctuations in the photon counting rate. With each of ten published critiques there was a reply from Frank *et al.*, in which the CLO was re-engineered to avoid detection, say by changing the mantle thickness, mean density or albedo, often in ways to make the CLO unlike any known terrestrial or astronomical object. It is surprising how well-specified the properties of the unseen CLO became when, by contrast, the density of real comets remains uncertain by an order of magnitude, even after the extensive observation of comet Halley by an international flotilla. The situation quickly became somewhat analogous to the case of Blondot, who, in 1903, was the only person able to observe the unique properties of the newly discovered N-rays⁷.

It is fair to ask whether the editor of *Geophysical Research Letters*, Alex Dessler, should have published the papers of Frank *et al.*, apparently against the advice of referees. The answer is far from

clear; the issue raised is not without interest. But by allowing individual replies to each criticism of the original idea, Dessler placed the burden of proof on critics to show that their objections were valid. The debate has thus been turned inside-out. The burden of proof should have been squarely on Frank *et al.* to examine other data sets or otherwise to find positive evidence for the existence of these objects.

This is precisely what Donahue *et al.*, in their paper in this issue, have done⁴. Previously, Donahue⁸ had shown that even at the low rate claimed by Frank *et al.* for the outgassing of water by the CLO, the number of hydrogen atoms produced by the dissociation of water by solar ultraviolet light would far exceed the known abundance of these atoms derived from many years of observations of the interplanetary Lyman- α glow⁹. But when he and his colleagues searched the records from the ultraviolet spectrometer experiments on Voyagers 1 and 2, they found that the concentration of hydrogen atoms between the orbits of Earth and Mars is indeed greater than that produced by the motion of the Solar System through the interstellar medium. By using a size distribution that matches the known rate of lunar cratering, Donahue *et al.* can produce the required hydrogen-atom abundance with 10 million times fewer CLOs per minute than Frank *et al.* and in so doing avoid many of the pitfalls in the hypothesis of Frank *et al.*.

The catch? These CLOs are the absolute inverse of the CLOs of Frank *et al.*. They have refractory cores surrounded by mantles of pure water ice. These, too, escape detection by ground-based astronomers, whose charge-coupled-device detectors would be sensitive to sunlight scattered from the smallest amount of dust impurities released by evaporation from a dirty surface (as happens with real comets). Moreover, the CLOs of Donahue *et al.* would account for all the lunar cratering, leaving no allowance for cratering by non-icy asteroidal debris.

The virtue of the analysis of Donahue *et al.* is that it is testable by instruments that are either available or that soon will be. The Galileo mission to Jupiter and its moons, postponed as a result of the Challenger accident, will reach the planet via a swing-by of Venus and two of Earth. Thus, the ultraviolet spectrometer on the spacecraft will be able to measure the Lyman- α emission between Venus and Jupiter. The most appropriate evidence would be the direct detection of a CLO or several CLOs, which would be difficult because of their proposed flux. In fact, Donahue *et al.* have deliberately described a CLO that would avoid detection by any of the several current search programmes for faint, slow-moving asteroids.

Fluorescent emission by hydroxyl (OH)

radicals, produced by the dissociation of water, could also reveal the CLOs. This emission, at a wavelength of about 310 nm, is produced by real comets¹⁰, and is more intense than Lyman- α emission, especially near the cometary nucleus. It is difficult, although not impossible, to see the fluorescence from Earth, because of attenuation by ozone in the atmosphere. NASA's comet rendezvous asteroid flyby mission now being planned will have cameras with narrowband OH filters that could be used to search for CLOs on the journey to the periodic comet Tempel-2.

What is the origin of CLOs, whatever their form? Presumably they are primordial remnants from the formation of the Solar System and share many of the known characteristics of the observed comets. It is difficult, however, to imagine that kilometre-sized comets were formed by aggregation of the CLOs proposed either by Frank *et al.* or by Donahue *et al.*. Anticipating this problem, Frank and his group have come up with a model of the Oort cloud which carefully segregates the different types of objects in the cloud, thus

allowing them to respond differently to different stellar perturbations. The bottom line of this model, which was presented at the recent meeting of the Division for Planetary Sciences of the American Astronomical Society (Pasadena, 10–13 November 1987), is the prediction of periodic extinctions of terrestrial flora and fauna by showers of the more massive members of the cloud. Is there any wonder that the community remains sceptical? □

1. Raup, D.M. & Sepkoski, J.J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 801–805 (1984).
2. Whitmire, D.P. & Jackson, A.A. *Nature* **308**, 713–715 (1984).
3. Davis, M., Hut, P. & Muller, R.A. *Nature* **308**, 715–717 (1984).
4. Donahue, T.M., Gombosi, T.I. & Sandel, B.R. *Nature* **330**, 548–550 (1987).
5. Frank, L.A. *et al. Geophys. Res. Lett.* **13**, 303–306 (1986).
6. Frank, L.A. *et al. Geophys. Res. Lett.* **13**, 307–310 (1986).
7. Seabrook, W. *Doctor Wood* (Harcourt Brace, New York, 1941).
8. Donahue, T.M. *Geophys. Res. Lett.* **14**, 213–215 (1987).
9. Ajello, J.M., Stewart, A.I., Thomas, G.E. & Graps, A. *Astrophys. J.* **317**, 964 (1987).
10. Feldman, P.D. *Science* **219**, 347–354 (1984).

Paul D. Feldman is Professor of Physics and Astronomy at the Johns Hopkins University, Baltimore, Maryland 21218, USA.

Ocean Drilling Program

Collisions in the Indian Ocean

*Leg 116 shipboard scientific party**

ON Leg 116 of the Ocean Drilling Program, we investigated the tectonic and sedimentological effects of the continental collision between India and Eurasia near the equator in the central Indian Ocean, over 3,000 km from the Himalaya collision zone (Fig. 1). We found that major uplift of the Himalayas had begun by the early Miocene, earlier than previously reported. We also investigated the timing and nature of faulting within a wide area of intraplate deformation and studied sediment sources and depositional processes on the distal Bengal Fan.

Continental collision began in the

Eocene with a 'soft' collision, probably between continental India and an island arc seawards of Asia^{1,2}. Convergence between the two continents continued, perhaps consuming a back-arc basin. The 'hard' continent-continent collision is thought to have begun later, resulting in the first main uplift of the Himalayas^{3,4}. Continued seafloor spreading at the Southeast Indian Ridge combined with growing resistance to shortening between India and Asia implies that the central Indian Ocean is under a large north-south compressive stress regime.

Classical plate tectonics assumes that large lithospheric plates behave as rigid units that deform only at their boundaries. The Indian-Australian Plate seems not to be behaving in this manner, rather it has been compressively deformed in its interior^{5–7}. The most spectacular and direct evidence from this deformation is the large-scale folding and faulting between the Chagos-Laccadive Ridge and the Ninetyeast Ridge and from 5° N to 10° S. Within the affected region, the oceanic crust and the overlying sediments are deformed into long-wavelength (100–300 km) folds with peak-to-trough amplitudes of 1–3 km. Superimposed on the long-wavelength undulations are faulted and deformed blocks, bounded by high-angle faults 5–20 km apart, which offset the top of the crust by up to 500 m (Fig. 2).

Rapid terrigenous sedimentation on the

*Co-chief scientists: James Cochran (Lamont-Doherty Geological Observatory, Palisades, New York) and Dorrik A.V. Stow (Department of Geology, Nottingham University). ODP Staff Scientist: Christian Aurox (Ocean Drilling Program, Texas A&M University, College Station). Also, Kazuo Amano (Department of Earth Sciences, Ibaraki Univ.), Peter S. Balson (British Geological Survey, Keyworth), Jacques Boulegue (Laboratoire de Géochimie, Univ. Pierre et Marie Curie), Garrett Brass (Univ. Miami), Jeffrey Corrigan (Dept. Geological Sciences, Univ. Texas, Austin), Stefan Gartner (Dept. Oceanography, Texas A&M University), Stuart Hall (Dept. Geosciences, Univ. Houston), Silvia Iaccarino (Istituto di Geologia, Univ. Parma), Toshio Ishizuka (Ocean Research Institute, Univ. Tokyo), Irena Kaczmarek (Mount Allison Univ. Sackville, New Brunswick), Heidmarie Kassens (Geologisch-Palaeontologisches Institut, Univ. Kiel), Gregory Leger (Dept. Oceanography, Dalhousie Univ.), Franca Proto Decima (Istituto di Geologia, Univ. Padova), Venkata Challa-gundla Raman (Dept. Geology, Andhra Univ., Visakhapatnam), Will Sager (Dept. Oceanography, Texas A&M Univ.), Koza Takahashi (Woods Hole Oceanographic Inst.), Thomas Thompson (580 Euclid Avenue, Boulder), Jean-Jacques Tiercelin (Oceanologie et Géodynamique, Univ. Bretagne), Mark Townsend (Dept. Geology, Univ. Nottingham), N.P. Wijayananda (Oceanography Unit, Natn. Aquatic Resources Agency, Colombo), Andreas Wetzel (Geologisches Inst., Tübingen), Colin Williams (Lamont-Doherty Geological Observatory).

incipient Bengal Fan began in the Eocene as a response to the first interplate collision^{1,2} and has continued to the present, producing the world's largest submarine fan. The Bengal Fan is more than 2,500 km long and is at least 16 km thick under the northern Bay of Bengal. The sediment is 1.5–2 km thick under the distal part of the fan near the Leg 116 sites.

We investigated the timing and development of the intraplate deformation by drilling a pair of companion sites on one of the fault blocks that make up the tectonic fabric of the region (Fig. 2). Site 717 is in the thickest part at the axis of a syncline in the sediments between the faults. It was designed to obtain a complete section of sediments where the unconformity marking the onset of deformation seems to have become conformable on seismic-reflection records. Site 719 is part-way up the block in an area where the syn-deformation sediments are thinner.

Sediments at both sites consist almost entirely of fan turbidites. The sedimentary sections correspond closely, and many distinctive individual turbidites and turbidite sequences between the two sites can easily be correlated. Attenuation of the section between sites 717 and 719 seems to have occurred by the pinching out of beds and the thinning of individual turbidites. Some intervals show marked reduction of thickness, whereas others are less attenuated. But in general it seems that the process has continued steadily ever since the onset of faulting, and therefore motion on the fault has been gradual and fairly constant. The rate of motion may have increased slightly with time. We penetrated the seismic horizon marking the onset of deformation (A in Fig. 2) at all the Leg 116 sites, and we find it is about 7 Myr old. There has been about 350 m of

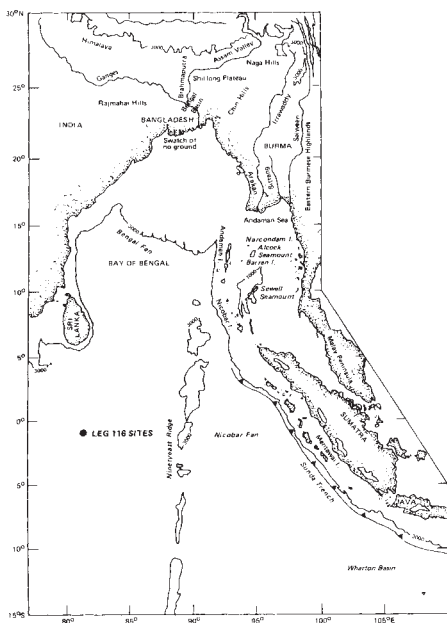


Fig. 1 Northeastern Indian Ocean, showing the location of the Leg 116 sites.

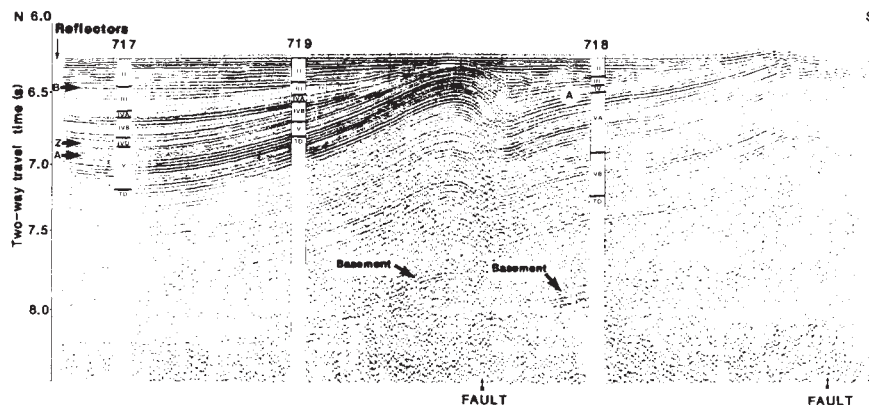


Fig. 2 Single-channel seismic reflection profile across the Leg 116 sites. Lithologic units are shown at location of sites with depth converted to two-way travel time. Unconformities A and B and reflector Z are noted to left of the profile. Total length of the seismic line, 18.5 km.

uplift across the fault, so that the average rate of motion has been about 50 m Myr⁻¹.

The limiting factor in determining the deformation history is the resolution that can be obtained on stratigraphic ages. Biostratigraphic control at all the Leg 116 sites is provided primarily by calcareous nannofossils, because the other microfossil groups are either absent or poorly preserved. But, subject to the resolution provided by the nannofossil zones, the data from sites 717 and 719 will allow us to determine the detailed history of the motion of the fault block. This will in turn provide constraints for theoretical modelling of the deformation process.

These two sites, particularly site 717, where the section appears complete, also yield a record of sedimentation on the distal Bengal Fan since the late Miocene, showing the nature, thickness and vertical succession of turbidites that have been transported as much as 2,500 km from the head of the fan. A thin layer of mud and clayey ooze overlies a sequence dominated by micaceous silty turbidites which accumulated very rapidly during the late Pleistocene at greater than 350 m Myr⁻¹ (Fig. 2, Lithologic unit II). This region constitutes a distinctive seismic stratigraphic unit which, in places, truncates lower reflectors. These relatively coarse-grained, rapidly deposited turbidites probably reflect the Quaternary uplift of the Himalayas, which provided a massive source of sediments. The silty turbidites overlie a thick section of mainly mud turbidites and thin interbedded pelagic clays that accumulated at a slower average rate of about 70 m Myr⁻¹ from the late Miocene to early Pliocene (Fig. 2, Lithologic units III and IV). We have tentatively identified at least three distinct sources of turbidites: silts and muds from the Ganges–Brahmaputra delta, which constitute the largest component; dark grey organic-rich muds with up to 5 per cent terrigenous plant debris from the upper slope of the Bay of Bengal; and almost white, carbonate-rich, biogenic turbidites, probably from a local seamount.

Site 718, on the next fault block south

from sites 717 and 719, where the post-Miocene section is greatly attenuated (Fig. 2), provided an opportunity to probe further back into the sedimentary history of the distal Bengal Fan. At this site, we obtained a sedimentary section to a depth of 960 below the sea floor, penetrating 775 of the approximately 1,200 m of pre-deformation sediment. One surprising result is the age and thickness of the fan. At the base of the hole, fan sediments of early Miocene age (17 Myr old) were still being penetrated with no evidence of approaching the base of the fan. Almost the entire Miocene section consists of silt and silty mud turbidites. Intervals up to 20 m thick of reddish brown pelagic clay and thin greenish silt-clay turbidites occur below a depth of 600 m below the sea floor. These intervals represent periods of slowed sedimentation and, possibly, the influx of turbidites from a different source such as the eastern Indian or Sri Lankan margin. The average sedimentation rate was about 70 m Myr⁻¹ throughout the Miocene. The Bengal Fan was well established at the Leg 116 sites (2,500 km from the Ganges delta) by the early Miocene and has been receiving sediment, including wood fragments and even coarse sand-sized grains ever since, suggesting that the main uplift of the Himalayas occurred earlier than is generally assumed.

A second surprise came from downhole temperature measurements. Site 718 is on an area of high local heat-flow to enable study of the effects of high heat flow on sediment and of hydrothermal circulation. We found dramatic evidence of vigorous hydrothermal circulation in the form of a temperature inversion. Temperatures in the silty turbidites of Lithologic unit II were scattered and were actually higher than in the upper part of underlying clay turbidites of unit III. Warm water appears to be rising up the fault to the north of site 718 (Fig. 2) and to be spreading laterally through permeable layers in the upper silty turbidites. At the same time, cooler sea water must be flowing downwards through silt layers within the clayey turbidites, which appear on seismic records

to crop out several kilometres to the south at the tip of the fault block. Our conclusions are supported by geochemical studies of interstitial waters, which show the effects of mixing between two end-members, one of which is sea water and the other a water chemically altered by basement rocks or by diagenetic processes.

Several factors influence sedimentation on the distal Bengal Fan. A primary control is the uplift history of the Himalayas, which are the main source of the sediments. This is seen, for example, in the pulse of coarser-grained silty turbidites in the Pleistocene following a significant phase of mountain building. A second influence is variations in sea level: a sharp rise in sea level near the Miocene/Pliocene boundary⁸ could have contributed to the change from silty to more muddy turbidite

deposition at that time. A third influence is tectonic activity related to the intraplate deformation. The block containing site 718 has been elevated relative to the block containing sites 717 and 719 (Fig. 2), and as a result the post-Miocene sedimentary history of the two blocks differs considerably. Normal fan processes such as channel migration and lobe switching have also influenced sedimentation. □

1. Curry, J.R. & Moore, D.G. *Bull. geol. Soc. Am.* **82**, 563–572 (1972).
2. Curry, J.R. *et al.* in *Ocean Basins and Margins* (eds Nairn, A.E.M. & Stehli, F.G.) Vol. 6, 399–450 (Plenum, New York, 1972).
3. Gansser, A. *The Geology of the Himalayas*, (Wiley-Interscience, New York, 1964).
4. Powell, C.M.A. & Conaghan, P.J. *Earth planet. Sci. Lett.* **20**, 1–12 (1973).
5. Eittreim, S.L. & Ewing, J. *J. geophys. Res.* **77**, 6413–6421 (1972).
6. Weissel, J.K. *et al.* *Nature* **287**, 284–291 (1980).
7. Geller, C.A. *et al.* *J. geophys. Res.* **88**, 1018–1032 (1983).
8. Haq, B.U. *et al.* *Science* **235**, 1156–1167 (1987).

Molecular endocrinology

Growth-hormone receptor cloned

Michael Wallis

LIKE most polypeptide hormones and growth factors, pituitary growth hormone (GH) acts by binding to plasma membrane-associated receptors on its target cells. But the mechanisms by which the GH-receptor complex then exerts intracellular effects are poorly understood. GH receptors have been characterized¹ in some detail from several tissues, but the field is a complex one because of species specificity¹, receptor heterogeneity^{2,4}, and the presence of soluble GH-binding proteins in both cytosol and plasma^{3,7}. The paper by Leung *et al.* on page 537 of this issue⁸ should help to resolve many of these issues. These authors have cloned complementary (c)DNA encoding GH receptors from rabbit and human liver, have obtained expression of the cDNA clones in cultured monkey COS-7 cells, and show that the amino-terminal sequence of the GH-binding protein in rabbit serum is very similar to that of the membrane-bound receptor in liver.

The authors purified detergent-solubilized GH receptor from rabbit liver and characterized it by SDS polyacrylamide gel electrophoresis, revealing a single main polypeptide of M_r about 130,000, considerably greater than that found previously using crosslinking studies¹. Leung *et al.* synthesized an oligonucleotide probe which they used to identify clones in a rabbit liver cDNA library. From these they determined the sequence of a full-length cDNA corresponding to GH receptor messenger (m) RNA, from which they showed that the amino-acid sequence of the receptor comprises 620 residues plus an 18-residue signal peptide (see figure). A hydrophobic region from residues 247–270 probably

represents a transmembrane domain, leaving 246 residues at the amino-terminal end as an extracellular domain, which presumably binds GH, and 350 residues on the cytoplasmic side of the plasma membrane, presumably involved with signal transduction. The M_r derived from the sequence (~65,000) is less than that observed for the receptor purified from liver, which can be largely accounted for by glycosylations (there are five potential glycosylation sites in the extracellular domain). Some of the liver-derived receptor molecules are linked to ubiquitin (M_r ~9,000), a protein that may be involved in regulation of receptor turnover.

Using fragments of the rabbit cDNA as probes, Leung *et al.* identified clones in a human liver cDNA library, from which they derived the sequence of a full-length cDNA for a putative human GH receptor. The human and rabbit receptors are very similar, showing about 84 per cent identity in amino-acid sequence; this homology is considerably greater than shown by the corresponding GHs, and is surprising in view of the species specificity of biological responses to GH.

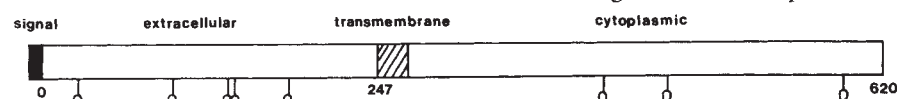
To establish whether the cDNA sequences obtained correspond to the complete receptors, Leung *et al.* constructed expression plasmids containing the full coding sequence of the rabbit receptor and a coding sequence for the extracellular domain of the human

receptor, and introduced them into monkey COS-7 cells, which normally do not express GH receptors. They found expression of a membrane-associated GH-binding protein in cells transfected with the rabbit receptor cDNA and secretion of a soluble GH-binding protein by cells transfected with human receptor cDNA. These binding proteins have specificities similar to those of receptors derived from normal tissues in that the rabbit protein can bind human GH, bovine GH and to a lesser extent ovine prolactin, whereas the human protein can bind human GH but not bovine GH or ovine prolactin. This observed species specificity for receptor binding is very similar to that seen in the biological actions on growth promotion, providing strong evidence that the cDNAs cloned correspond to receptors that mediate the growth-promoting effects of GH.

Leung *et al.* also show that the amino-terminal sequence of the soluble GH-binding protein found in rabbit serum is identical to that of the GH receptor in liver. The soluble binding protein probably represents the extracellular domain of the receptor produced either by proteolytic cleavage or altered processing of a precursor of the receptor mRNA. The studies on cDNAs do suggest that some of the mRNA in liver could encode a variant form of the receptor lacking most of the intracellular domain, but it is not clear whether this is the secreted binding protein.

The work of Leung *et al.* will have a significant impact on our view of the GH receptor. It provides a definitive sequence for the membrane-bound GH-binding proteins in rabbit and human liver and demonstrates that the differences between species are less than previously suspected. This single polypeptide (after glycosylation) is all that is needed for GH binding, although it is not yet established whether other subunits are needed for biological activity. It is likely that these binding proteins really are the biological receptors for the growth-promoting actions of GH, because the species specificity for hormone binding clearly matches that for biological activity. The paper also clarifies the relationship between the GH receptor and the GH-binding protein in serum. It provides few clues, however, about the mechanism of signal transduction. The receptor sequence is not related to known tyrosine-kinase growth-factor receptors, or indeed to any other known protein.

The cloning of the GH receptor should



The putative growth-hormone receptor precursor is a polypeptide chain of 620 residues plus an 18-residue signal peptide. A hydrophobic region (residues 247–270) probably constitutes a single transmembrane domain. Eight asparagine residues (circles) are potential glycosylation sites.

provide a valuable basis for further studies on the action of the hormone. Expression of the cDNA in eukaryotic cells means that precise mutations can be introduced to allow study of the mechanism whereby GH binds to the receptor and, if introduction of the receptor can be linked to biological activity, to allow studies on the intracellular events that follow GH binding. Investigation of hormone-receptor interactions will be facilitated by the recent report⁹ of the three-dimensional structure of porcine GH; more than half the sequence of the protein is in the form of four α -helices, arranged in a tightly packed antiparallel helical bundle. At a recent conference, J. Schwartz reported that binding in mouse adipose cells is found to be associated with increased receptor phosphorylation, which may be a clue to the events that follow binding of GH to its receptor.

The receptor sequence reported by Leung *et al.* again raises the question as to how binding of hormone to the extracellular domain of the receptor leads to transmission of a signal to the cytosolic

domain via a single, short transmembrane sequence. Hormone binding may induce aggregation of receptors moving laterally in the fluid plasma membrane, as has been suggested¹⁰ for some other growth-factor receptors. There seems every possibility that the report of Leung *et al.*, together with the other recent developments discussed here, will spark a renewed interest in the poorly understood mechanisms involved in transducing the signal provided by GH and related hormones. □

1. Hughes, J.P. *et al.* *Polypeptide Hormone Receptors* (ed. Posner, B.I.) 157–199 (Dekker, New York, 1985).
2. Barnard, R. *et al.* *Biochem. J.* **231**, 459–468 (1985).
3. Ymer, S.I. & Herington, A.C. *Biochem. J.* **242**, 713–720 (1987).
4. Thomas, H. *et al.* *Biochem. J.* **243**, 365–372 (1987).
5. Ymer, S.I. *et al.* *Biochem. J.* **221**, 617–622 (1984).
6. Ymer, S.I. & Herington, A.C. *Molec. cell. Endocr.* **41**, 153–161 (1985).
7. Baumann, G. *et al.* *J. clin. Endocr.* **62**, 134–141 (1986).
8. Leung, D.W. *et al.* *Nature* **330**, 537–543 (1987).
9. Abdel-Meguid, S.S. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **84**, 6434–6437 (1987).
10. Yarden, Y. & Schlessinger, J. in *Growth Factors in Biology and Medicine* (eds Evered, D., Nugent, J. & Whelan, J.) 23–40 (Pitman, London, 1985).

Michael Wallis is in the School of Biological Sciences at the University of Sussex, Falmer, Brighton BN1 9QG, UK.

Neural networks

Implementation and analysis

Geoffrey North

NEURAL networks have become the subject of intense interest in both pure and applied research. So much was clear at a recent meeting* at which a diverse collection of computer scientists, electronic engineers, biologists, physicists and mathematicians heard talks ranging from how model neural networks can be implemented by real devices, to what can be rigorously proved about their computational capabilities. The hope is that the study of model neural networks will not only deepen understanding of how the brain works but also revolutionize computing, especially by the development of devices for the kind of information-processing task we are so good at and which conventional computers find so difficult.

As I reported in a recent News and Views article¹, the resurgence of interest in neural networks has been stimulated by theoretical developments in how networks can learn. Hitherto, the teaching and operation of neural networks have been simulated on conventional serial computers, with the result that the parallel nature of the algorithms has not been exploited, so that the operations are much slower than they need be. There were two reports at the meeting (N.H. Brown, MRJ/Perkin-Elmer; S. Tomboulis, NASA Langley Research Center) of how

the parallel computer called the 'connection machine'² can be programmed to simulate a neural network. Although such an approach is likely to have the flexibility desired by pure researchers, it is unlikely to be the way forward for real-world applications of neural networks.

Accordingly, attempts are being made directly to implement electronic and optical analogues of neural-network models. For example, J. Alspector (Bell Communications Research) has designed a true 'learning chip' based on the neural network of Hinton and Sejnowski³ called the 'Boltzmann machine' — a stochastic generalization of a Hopfield network. A Hopfield network consists of a set of units, representing neurons, each of which is connected to every other unit by connections with variable, but symmetrical, weights. Hopfield had earlier shown⁴ that, in such a network, a simple update rule always leads to a decrease of energy of the network towards a local minimum of the energy surface, which collectively are the 'memories' of the network, acquired by a simple learning rule for adjusting the connection weights. The same kind of network can be applied to optimization tasks, but the trouble is that the network can get trapped at non-global minima of the energy surface.

Hinton and Sejnowski avoid this problem by simulated annealing (as used, for example, in the theory of spin-glasses) in

which the update rules for learning and working of the network include statistical noise governed by a parameter called 'temperature', by analogy with thermodynamics. This causes the system occasionally to take uphill steps on the energy surface which allows it to escape from poor local minima. If the network is allowed to reach 'thermal equilibrium' there is a simple learning procedure that changes the weights so as to make the whole network better at performing a desired input-output mapping. Each weight change depends only on the behaviour of the two units the weight connects, and this locality makes it easy to implement the learning procedure on a chip.

In Alspector's chip (see figure), which is soon to be fabricated, the 'neurons' are implemented by amplifiers which are connected by 'adaptive synapses' — symmetrical variable resistors, the states of which are individually controlled by local 'black boxes' that, in the learning mode, count correlations in the activities of the connected neurons when the network is at thermal equilibrium. These counts are used to increase or decrease the synaptic weights in the way specified by the Boltzmann-machine learning procedure. The stochasticity comes from an electronic noise generator.

The chip is designed to implement supervised learning in which, for every input, the network is told what should be the correct output, but with minor modifications it could also implement unsupervised and 'reinforcement' learning. In this last type of learning the chip is not given the correct outputs, but a 'critic' tells it whether its outputs are 'good' or 'bad'. Learning embedded in this VLSI (very-large-scale integrated circuit) chip is about a million times faster than with a VAX serial computer.

Much interest was aroused by the publication last year⁵ of the 'back-propagation' algorithm for teaching multi-layer networks. Back-propagation works by sending error signals back through the network, and adjusting the connection weights so as to reduce the difference between the actual and desired output of the network. Not only is this biologically a somewhat implausible learning mechanism, it is also difficult to implement electronically. According to D. Psaltis (Caltech), however, back-propagation can be implemented by an optical network. Psaltis described such a network in which the 'neurons' consist of liquid crystal light valves which carry out a threshold operation — if their input exceeds some threshold they give a '1' output, otherwise they output '0'. These 'neurons' can be densely packed as a two-dimensional array, fully interconnected by connections both specified and effected by holograms.

Psaltis explained how such a network

* IEEE conference Neural information processing systems — natural and synthetic Denver, Colorado, 8–12 November 1987.

can operate in two modes: (1) as a Hopfield network, functioning as a content-addressable memory where multiple cycles of light around the system lead to the selection of some stable state — a 'memory' — if the input was within the 'basin of attraction' of some memory of the network; and (2) as a multi-layer back-propagation network, where an error image is generated and sent back through the system, the holographic connections being adjusted as a result just as in the back-propagation algorithm.

Perhaps the most promising applications of neural networks, at least in the short term, are as 'real-time' sensory processing devices. Thus Carver Mead (Caltech) reported on an analogue circuit, on VLSI scale, that processes sound information in real time in a way that closely simulates cochlear processes; will this 'artificial cochlea' provide new aids for the deaf? Christof Koch (Caltech) described an analogue circuit for use in the perception of visual motion, designed to implement a 'regularization algorithm'⁶ that uses a smoothness constraint to avoid the ambiguity in the motion vectors of visual contours seen locally, together with a way of detecting likely discontinuities so as to prevent inappropriate 'over-smoothing'. With Mead's help, Koch hopes to fabricate his design.

A wide range of applications of neural network models were reported at the meeting, ranging from the control of a two-joint robot arm (a difficult problem in 'inverse dynamics') to the playing of backgammon. A. Lapedes (Los Alamos National Laboratory) described a particularly revealing application to time-series

analysis. The problem is to predict the future development of a system variable from a sample of its past values taken at discrete times, a difficult task if the behaviour is chaotic, as it may be for relatively simple non-linear dynamical systems. Lapedes reported that multi-layer networks cope remarkably well. The data are given to the network in the form of x_t/x_{t+1} input/output pairs, followed by back-propagation learning. It turns out that the network can often discover the function underlying the development of the system, even when it is quite complicated. The network carries out a kind of Fourier analysis of the function, but in terms of the sigmoid input-output functions of the network units rather than the usual trigonometric functions. The function can be recovered from the connection weights of the educated network, which are the coefficients in its series representation.

Theoreticians at the meeting addressed such questions as how learning can be speeded up, and what can be rigorously proved about the properties of networks. A wide range of theoretical frameworks are being brought to bear on these problems: information theory, complexity theory, coding theory and dynamical systems theory, to name but a few. Several talks aimed at generalizing existing learning algorithms in various ways.

Thus D. Parker (Menlo Park, California) showed that back-propagation learning can be speeded up if the descent of the error surface in weight-space is guided by the second derivative of error with respect to the weights, rather than just using the first derivative (which just

gives the local path of steepest descent). And S. J. Hanson (Bell Communications Research) explained how the error function can be varied from the usual least-squared function — a lower power, for example, decreases the effect of 'noise'.

Yet another variant is to use 'higher-order' networks, where connections are defined between three or more neurons rather than the usual two: according to L. Giles (AFOSR/NE Bolling Air Force Base), the computational power and speed of learning of a network can be increased in this way, but only at the cost of increased specialization. Perhaps more significant is the introduction by F. Pineda (Johns Hopkins University) of a learning rule that generalizes back-propagation to networks with feedback: based on an analysis considering the network as a dynamical system, Pineda's technique gets round the problem of the oscillations to which feedback networks are prone.

S. Venkatesh (University of Pennsylvania) addressed the general questions: given a function, what can be said of the complexity of a network that can implement the function? And what can a given network do? He has proved that for a given number of neurons it is computationally more efficient to arrange them as a shallow, broad network (in other words with a relatively large number of neurons per layer) than a deep, narrow network (with a relatively large number of layers).

At a more general level a recurring theme at the meeting was the importance of 'representation': how information is represented in the network by the pattern of activity of its units. The importance of representation was emphasized by T. Sejnowski (Johns Hopkins University) in his plenary lecture, and was clearly illustrated in the case of the network for playing backgammon by G. Tesauro (University of Illinois). Although the average performance is good, the network has a tendency to make bad mistakes in certain situations, one type of which Tesauro has pinned down to inappropriate generalization during learning; this can be avoided by changing the representation of expert knowledge used to teach the network.

It is clear that the study of neural networks is at an exciting stage. While many speakers in Denver were quick to point to early precedents for some of the current ideas, the field is still in its infancy — but the interest it has already aroused should ensure that future progress is rapid. □

1. North, G. *Nature* **328**, 107 (1987).
2. Hillis, W.D. & Barnes, J. *Nature* **326**, 27 (1987).
3. Ackley, D.J., Hinton, G.E. & Sejnowski, T.J. *Cognitive Science* **9**, 147 (1985).
4. Hopfield, J.J. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2554 (1982).
5. Rumelhart, D., Hinton, G.E. & Williams, R. *Nature* **323**, 533 (1986).
6. Poggio, T., Torre, V. & Koch, C. *Nature* **317**, 314 (1985).

Geoffrey North is Deputy Biology Editor of *Nature*.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

A computer-generated image of a silicon chip, 6.5×6.5 mm, designed to implement the 'Boltzmann-machine' system of neural network learning. The 'neural network' is in the lower left quadrant of the chip. (Courtesy of J. Alspector.)

Pulsars

Polarized γ -rays from Vela

A. J. Dean

INCREASINGLY sophisticated observations of pulsars over the past two decades have led convincingly to the conclusion that pulsars are magnetized neutron stars. But despite the wealth of detail accumulated, there is still no comprehensive model of the pulsation mechanism, because of the lack of knowledge of the configuration of the magnetic field and particle flux. Now, P.A. Caraveo *et al.* (*Astrophys. J.*, in the press) report that the flux of 100-MeV γ -rays from the Vela pulsar are polarized. This result is of great significance because of the close link between photon polarization and the ordering of the source. These new observations open the way to a better understanding of pulsar physics.

Because the wavelength of γ -rays is so short (about 10^{-12} m at 100 MeV), classical wave techniques cannot be used to measure their polarization. The kinematics of the particles produced by the interactions of γ -ray photons (Compton scattering and 'pair production') can be used, however. The emerging particles (electrons or electron-positron pairs) have azimuthal distributions related to the plane of the electric vector (polarization) of the incident γ -ray.

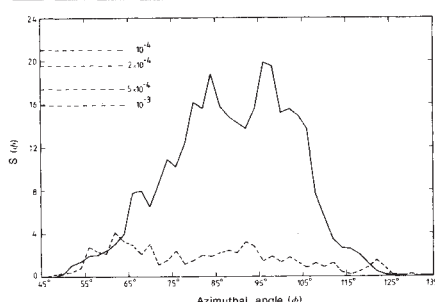
The means of studying γ -ray polarization by the pair-production process ($\gamma \rightarrow e^+ + e^-$) in spark chambers was recently described by A.A. Koslenkov and I.G. Mitrofanov (*Sov. Astr.* **29**, 591–593; 1985), who based their work on the basic theory developed by L.C. Maximon and H. Olsen (*Phys. Rev.* **126**, 310–319; 1962). The azimuthal distribution of the planes containing the electron-positron pairs is related to the direction of the electric vector of the incident, linearly polarized γ -rays. This is seen as a broad quadrupolar modulation of the distribution of produced pairs about the azimuthal angle ϕ relative to the incident photons and provides a means of investigating the degree of polarization of incident γ -ray fluxes.

As well as the intrinsic, azimuthal spread, two other effects broaden the distribution further. First, pair production is a three-body process that occurs in the presence of a nucleus which recoils, introducing an unknown perturbation. Second, the electron and positron are Coulomb-scattered as they emerge from the conversion material of the spark-chamber plates. The net result is a reduction in the depth of modulation.

Caraveo *et al.* apply this theory to data previously obtained at the European Cos-B satellite γ -ray telescope. The spark

chamber within the instrument identifies the trajectories of the two materialized electrons in some detail, so that a three-dimensional reconstruction of individual events is possible. Only γ -ray events that have clearly defined tracks of the two electrons in the form of an inverted V are analysed. A Monte-Carlo simulation of the Cos-B configuration shows that the signal from completely polarized flux would be modulated by about 20 per cent, and that at least 2,000 events would be required to give a statistically meaningful result.

Emission from the Vela pulsar, which was chosen because it has provided more



The azimuthal modulation of the plane containing the electron-positron pairs derived from 2,526 pulsed Vela γ -rays that materialize in the Cos-B spark chamber (solid curve). The dashed curve applies to 3,109 photons from the Cygnus region used as a comparison. S is the deviation function as defined in Caraveo *et al.* Overall chance occurrence probability levels are shown as derived from Monte-Carlo simulations.

suitable and pulsed γ -ray events (2,526) than any other source detected by Cos-B, is strongly linearly polarized (see figure). Two further tests confirm this result. First, the azimuthal modulation is only apparent if the data are transformed from the coordinates of the spin-stabilized satellite to the 'despun' coordinates of the source. Second, γ -rays from the Cygnus region of the sky, of similar statistical quality, show no polarization, which is as expected, given the extended nature of the source.

Thus Vela seems to be a strongly polarized source and, by implication, it is a naked neutron star. It is probable that the polarized γ -rays are emitted by either curvature or synchrotron radiation. Also the γ -ray source must be sufficiently close to the neutron star for the magnetic field to be well ordered, but not so close that photons are lost by pair creation as they traverse suitably intense magnetospheric field lines.

The polarized photons are strongly correlated with the Vela pulsar rotation. The broad spread (30°) in the azimuthal distribution, however, precludes the possibility of identifying any relation between the phase of the pulsar and a swing in the direction of polarization of the γ -rays (as found in radio signals). But the degree of polarization does seem to vary throughout the pulse cycle, and this may help to elucidate the geometrical configuration of the pulsar. Separate analyses of the two main pulses, the interpulse region, and the background (emission in the period between the secondary and primary pulses) show that the interpulse is almost completely polarized, whereas the two pulses are, at best, only weakly polarized and the background is completely isotropic.

This result is compatible with the geometrical model proposed by F.G. Smith (*Mon. Not. R. astr. Soc.* **219**, 729–736; 1986) to explain all the emission (radio to γ -ray) of the Crab and Vela pulsars for which the interpulse emission is derived from curvature radiation in a fan beam. The lack of polarization in the first pulse is probably related to curvature radiation from the polar region, which is rotated by 360° (and thus smeared) with respect to the line-of-sight.

A similar analysis of γ -rays from the Crab (1,533 suitable events) and Geminga (1,204 suitable events) failed to produce a significant result. Clearly the poor statistical quality, combined with instrumental effects that give a low depth of modulation, renders a meaningful result unlikely. In the case of the Crab, any polarization is further stifled because half of the γ -rays emission is unpulsed and must originate from the Nebula or from a region that is not ordered by the magnetic field of the neutron star. It is a pity that there is no definitive result for Geminga. A positive detection of linear γ -ray polarization would confirm the compact nature of this object, which, because of the implied magnetic field, could then be identified as a neutron star rather than, say, a black hole.

The Cos-B investigation institutes a useful technique for the study of neutron stars and other objects. The close connection between the polarization of γ -rays and their parent particles and fields gives observers an undistorted view of the source configuration. Cos-B is hampered by poor statistics and a design not intended for polarization studies. Forthcoming telescopes, COMPTEL and EGRET, with improved data collection, and GRASP (γ -ray spectroscopy and positioning), with built-in polarimetry capability, will allow routine, accurate measurements of γ -ray polarization. $\square$

A.J. Dean is in the Department of Physics, University of Southampton, Southampton SO9 5NH, UK.

More approaches to the travelling salesman guide

SIR—In David G. Bounds's interesting exposition¹ of several intriguing new approaches to combinatorial optimization, he is unfortunately and seriously misleading when he suggests that these new approaches "seem likely to lead to much better algorithms for finding acceptably low-cost minima" for the travelling salesman problem (TSP). The Lin–Kernighan algorithm², well-known to researchers in computer science and operations research but apparently not to Bounds or many of the authors of his references, substantially outperforms all these approaches, both in speed and the quality of solution found.

The Lin–Kernighan algorithm is a sophisticated successor to the naive 3-OPT algorithm mentioned by Bounds. It finds better tours, and is orders of magnitude faster. It has been widely implemented, and is well-known to be practical for instances substantially larger than those discussed by Bounds. For instances consisting of points in the plane under the euclidean metric, our own implementation of Lin–Kernighan finds tours that are within 2% of optimal for instances with as many as 50,000 cities. For 10,000 cities the running time is little more than an hour on a VAX 8550. For 100 cities, it is only 3 s.

The new approaches discussed by Bounds offer Lin–Kernighan little competition. Consider simulated annealing, and the type of instance studied by Kirkpatrick and Toulouse³ and cited by Bounds. For 400-city instances of this type, Kirkpatrick and Toulouse found tours of length about 2.4. Lin–Kernighan, on the other hand, finds tours with length 2 (and for each instance comes within 2–3% of the Held and Karp⁴ lower bound on optimal tour length²). Moreover, whereas Lin–Kernighan takes about 20 s to find these tours, we find simulated annealing requires more than 6 h.

In addition to simulated annealing, Bounds covers two biologically motivated approaches: the neural-net approach of Hopfield and Tank⁵ and the genetic approach of Brady⁶, and mentions a fourth approach based on an analogy with rubber bands⁷.

The biggest instance Hopfield and Tank considered was 30 cities. I provided them with this instance, and when I did so I also told them the length of the optimal tour, which can be generated in less than a second using Lin–Kernighan, and whose optimality can be verified in 3 s. In all their many neural-net simulations on this instance, Hopfield and Tank never came within 17% of the optimal tour length⁵.

Brady also worked on only a tiny instance (64 cities, where the best of 10 runs of Lin–Kernighan is likely to be optimal), and reports that taking the best of a

collection of random-start 2-OPT runs was essentially as good as any of his techniques involving mating⁶. Multiple-start 2-OPT is a well-known procedure, and is not competitive with Lin–Kernighan.

The best of these last three approaches appears to be that of Durbin and Willshaw⁷, who found an optimal solution to the 30-city instance mentioned above. For instances with $n = 100$, however, they apparently performed no better than 3-OPT, worse than Lin–Kernighan.

Note that for instances as small as those on which the last three approaches were tested, there now exist programs that routinely find verified optimal solutions in reasonable amounts of time. The best such optimization algorithm is that of Padberg and Rinaldi⁸, which regularly finds verified optima for 100-city instances in what would be about 5 min on a VAX 8550. (The largest TSP they have solved

was a real-world instance with 2,392 cities, requiring 27 h on a supercomputer.)

The TSP is an intriguing problem, and those of us who have been working on it for years welcome its new-found popularity and the consequent influx of new ideas and approaches. I hope that in future, however, the evaluation of these new approaches will more fully take into account the strong competition provided by the techniques already in use.

DAVID JOHNSON

AT&T Bell Laboratories,
600 Mountain Avenue,
Murray Hill, New Jersey 07984-2070, USA

1. Bounds, D.G. *Nature* **239**, 215–219 (1987).
2. Lin, S. & Kernighan, B.W. *Oper. Res.* **21**, 498–516 (1973).
3. Kirkpatrick, S. & Toulouse, G. *J. Phys., Paris* **46**, 1277–1292 (1985).
4. Held, R.M. & Karp, R.M. *Oper. Res.* **18**, 1138–1162 (1970).
5. Hopfield, J.J. & Tank, D.W. *Biol. Cyb.* **52**, 141–152 (1985).
6. Brady, R.M. *Nature* **317**, 804–806 (1985).
7. Durbin, R. & Willshaw, D. *Nature* **336**, 689–691 (1987).
8. Padberg, M. & Rinaldi, G. *Oper. Res. Lett.* **6**, 1–8 (1987).

Returns on boomerangs

SIR—When a boomerang is thrown, the far end has a positive velocity relative to the chord but the near end has a negative velocity and therefore a negative Reynolds number. If the blade is twisted so that when thrown right-handed it has wash-in or positive incidence at the far end it will return to the thrower. If it has wash-out or is thrown left-handed the aerodynamic forces will tend to balance out because the nearward travelling blade will have positive incidence. Under these circumstances, if thrown in the horizontal plane the boomerang will travel on a curve having a large radius, approximating to a straight line.

The determination of whether the boomerang described by Valde-Nowak, Nadachowski and Wolsan (*Nature* **329**, 436–438; 1987) would return could easily be decided by measurement of the incidence variation across the span. Determining whether the palaeolithic thrower was left- or right-handed would be more difficult.

BRENNIG JAMES

Cherry Orchard,
Marlow Common,
Buckinghamshire, UK

The burden of proof in linking AIDS to scrapie

SIR—We have questioned^{1,2} the significance of similarities noted by Haseltine and Patarca³ between the scrapie-associated protein, PrP 27–30, and the human immunodeficiency virus, HIV-1. Patarca *et al.*⁴ defend the significance of this sequence resemblance. This supposed relationship could affect directions of AIDS research; also, it calls into question the value of sequence similarity measures as evidence for ancestral or functional

relationships between molecules. We must therefore point out weaknesses in the arguments of Haseltine and colleagues.

The central question in sequence comparison is biological relevance, not statistical significance. Statistical significance is typically used as a clue to biological relevance, which is more difficult to assess. Patarca *et al.*⁴ maintain that probability values of 10^{-3} and 10^{-1} for the HIV–PrP comparison are significant. However, there are about 12,500 entries in the GenBank(R) sequence database. Thus, for the HIV–PrP alignments with a probability value of 10^{-3} , we can expect to find 12–13 unrelated sequences with similar comparison scores in a search of the database. Actual searches by one of us (J.F.B.) yielded 7 and 13 equally good or better alignments for HIV and PrP, respectively. Yet, it is unlikely that any of the aligned sequences are truly related to HIV or PrP; they come from genes as diverse as chloroplast ribosomal RNA and rat cytochrome P-450c.

Patarca *et al.*⁴ assert that Bazan *et al.*² used statistical scores from two algorithms that were not comparable. In fact, the program FASTN was only used to search GenBank for loci with sequence similarity to HIV or PrP. The statistical significance of each similarity was then evaluated using ALIGN.

PrP shows no similarity to visna virus, equine infectious anaemia virus or HIV-2 (ALIGN scores of 0.2 s.d., –2.1 s.d. and 0.5 s.d.), yet these viruses are related to HIV-1 (refs 5, 6). Patarca *et al.*⁴ dismiss this evolutionary comparison on the grounds that the viruses may have undergone different rates of sequence divergence which could obscure their relationship to PrP. We note that Haseltine and Patarca³ were the first to invoke an evolutionary argument when they used a visna virus–PrP alignment to corroborate their

theory of an HIV-PrP relationship. Furthermore, available sequence data allow the possibility of unequal rates to be excluded by relative rate tests⁷. For these reasons, the burden of proof of an HIV-PrP relationship still lies with Haseltine and colleagues.

MICHAEL J. BRAUN

University of Cincinnati,
Cincinnati, Ohio 45221, USA

MATTHEW A. GONDA

Frederick Cancer Research Facility,
Frederick, Maryland 21701, USA

DAVID G. GEORGE

National Biomedical Research

Foundation,

Georgetown University Medical Center,
Washington, DC 20007, USA

J. FERNANDO BAZAN

Department of Biophysics,

University of California,

Berkeley, California 94720, USA

ROBERT J. FLETTERICK

STANLEY B. PRUSINER

Departments of Biochemistry,

Biophysics and Neurology,

University of California,

San Francisco, California 94143, USA

1. Braun, M.J. & Gonda, M.A. *Nature* **325**, 113–114 (1987).
2. Bazan, J.F., Fletterick, R.J. & Prusiner, S.B. *Nature* **325**, 581 (1987).
3. Haseltine, W.A. & Patarca, R. *Nature* **323**, 115–116 (1986).
4. Patarca, R. *et al. Nature* **326**, 749 (1987).
5. Gonda, M.A. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 4007–4011 (1986).
6. Guyader, M. *et al. Nature* **326**, 662–669 (1987).
7. Sarich, V.M. & Wilson, A.C. *Proc. natn. Acad. Sci. U.S.A.* **58**, 142–148 (1967).

Biological regulation of climate

SIR—The requirement for a biological regulation of the climate, according to Charlson *et al.*¹, is first, that the source of most of the cloud-condensation nuclei (CCN) over the remote oceans is dimethylsulphide, and second, that the salt particles always found in the marine atmosphere make a negligible contribution to the CCN. They state that sea-salt particle concentrations "... at cloud height are typically not more than 1 cm^{-3} ...". We believe that the particle concentrations are at least an order of magnitude more.

The classic sea-salt particle-size distributions obtained by Woodcock² at cloud base altitudes as a function of wind speed do indeed show concentrations of only about 1 cm^{-3} , but his sampling method did not allow him to collect particles smaller than $2\text{-}\mu\text{m}$ radius as a droplet of seawater. However, it was clear from the slope of his cumulative curves that smaller salt particles existed. Later, with refinements to his sampling method³, he extended his size distributions down to nearly $0.2\text{-}\mu\text{m}$ radius and found salt particle concentrations of about 15 cm^{-3} . His earlier wind-speed dependent cumulative curves², if extrapolated, converge at a concentration of about 15 cm^{-3} . This

suggests that salt-particle concentrations of around 15 cm^{-3} , due to the long residence time of the small salt particles, will change little with changing wind speed.

There is further evidence for sea-salt particles contributing significantly to the CCN. From measurements along the shore in Hawaii, Blanchard⁴ deduced that the salt particle flux to the atmosphere in a breaking wave is about $4,000 \text{ cm}^{-2} \text{ s}^{-1}$. Recently, in laboratory experiments to model a breaking wave at sea, Cipriano *et al.*⁵ obtained nearly the same value, $3,500 \text{ cm}^{-2} \text{ s}^{-1}$. Assuming that only 0.2 of these were large enough to be activated as CCN at 0.5 per cent supersaturation in clouds, and making other reasonable assumptions for the per cent whitecap coverage at sea and particle residence time, Cipriano *et al.*⁵ calculated that the measured flux was sufficient to produce concentrations of sea-salt CCN of 18 cm^{-3} in the lowest kilometre of the atmosphere.

Finally, measurements made from aircraft by Dinger *et al.*⁶ in the trade winds near Puerto Rico of the volatility of the CCN showed that above the temperature inversion all CCN are volatile, but below the inversion the fraction of CCN that is non-volatile increased as they approached the sea. Concentrations of at least 20 cm^{-3} were found at cloud base altitudes, and they assumed this to be sea salt.

It may be true that the source of most of the CCN is dimethylsulphide, but with a steady-state background concentration of sea-salt particles of $15\text{--}20 \text{ cm}^{-3}$, all of which can serve as CCN, a biological regulation of the climate is less obvious.

DUNCAN C. BLANCHARD

RAMON J. CIPRIANO

Atmospheric Sciences Research Center,
State University of New York at Albany,
Albany, New York 12222, USA

1. Charlson, R.J., Lovelock, J.E., Andreae, M.O. & Warren, S.G. *Nature* **326**, 655–661 (1987).
2. Woodcock, A.H. *J. Meteor.* **10**, 362–371 (1953).
3. Woodcock, A.H. *J. geophys. Res.* **77**, 5316–5321 (1972).
4. Blanchard, D.C. *J. rech. Atmos.* **4**, 1–6 (1969).
5. Cipriano, R.J., Monahan, E.C., Bowyer, P.A. & Woolf, D.K. *J. geophys. Res.* **92**, 6569–6576 (1987).
6. Dinger, J.E., Howell, H.B. & Wojciechowski, T.A. *J. atmos. Sci.* **27**, 791–797 (1970).

CHARLSON AND WARREN REPLY — We wish to make four points. First, regulation of climate by CCN from biologically generated dimethylsulphide does not require "that the salt particles always found in the marine atmosphere make a negligible contribution to the CCN", as stated by Blanchard and Cipriano. Our hypothesis requires only that the biologically produced CCN from dimethylsulphide alters the particle number concentration sufficiently to produce climatologically important shifts in albedo. Changes of only 30 per cent in the CCN numbers appear to be important to climate.

Second, we do not wish to enter into a debate as to whether the sea-salt CCN concentrations in clouds are 1 or 10 cm^{-3} .

What is more important is what fraction of the measured total droplet concentration of $30\text{--}200 \text{ cm}^{-3}$ is nucleated on sea-salt and what fraction is nucleated on dimethylsulphide-derived sulphate. Even if sea-salt CCN concentration was 20 cm^{-3} (which we believe is too high) it appears that sulphate CCN is still present in sufficient abundance to control the overall droplet concentration and hence the albedo.

Third, we chose to reference the excellent review of data on CCN, sea-salt and sulphate number concentrations by Pruppacher rather than to lengthen an already long paper. We certainly would have included the pioneering work of Woodcock, Blanchard and others had we had the space. We too believe the preponderance of opinion is that the fraction of droplets nucleated by sea-salt is small.

Finally, we believe that Blanchard and Cipriano would concur that more data on actual CCN composition and concentrations would help to refine the quantitative aspects of this intriguing hypothesis.

ROBERT J. CHARLSON

STEPHEN G. WARREN

Department of Atmospheric Physics,
University of Washington,
Seattle, Washington 98195, USA

Contributions to a quest

SIR—My principal concern with the News and Views article entitled "The end of the quest" by Paul M. Vanhoutte (*Nature* **327**, 459–460; 1987) is his omission of reference to the pertinent research conducted in this laboratory which provides strong experimental evidence that endothelium-derived relaxing factor (EDRF) is nitric oxide (NO). Instead, only Robert F. Furchgott is referenced as having proposed that EDRF may be NO. The reference quoted was a monograph to be published in *Mechanisms of Vasodilation* (ed. Vanhoutte, P.M.; Raven, New York), which represents a talk that was delivered at a symposium held in Rochester in July 1986. What is disconcerting about Vanhoutte's omission of our own contribution is that we also have a monograph to be published in the same book. This represents a talk that I delivered at the same symposium. Our monograph specifically addresses the close similarities in the biological properties of EDRF and NO, and contains our hypothesis that EDRF is NO or a closely related nitroso species. As Vanhoutte organized the symposium and edited both monographs, it is particularly distressing to us that fair and proper recognition was not given to our major contributions in this exciting area of research.

LOUIS J. IGNARRO

Department of Pharmacology,
UCLA School of Medicine,
Center for the Health Sciences,
Los Angeles, California 90024, USA

Steppes for conservation

Algirdas Knystautas

Environmental Policy in the USSR. By Charles E. Ziegler.
Frances Pinter, London/University of Massachusetts Press:1987. Pp.195. £22.50, \$35.

It is ironic that, just as this review was being written, *Izvestiya*, one of the two principal daily newspapers in the Soviet Union, reported on a meeting of the Politburo of the Communist Party of the Soviet Union, which was entirely devoted to environmental affairs. The Politburo is, of course, a policy-making body. And Ziegler's book is entirely devoted to policy.

Conservation-minded people in the Soviet Union are happy with the fact that the state owns all the land, water and other natural resources. This ought to work admirably, because, it would appear, there is only a single owner responsible for protection of the natural environment. There is no need to buy land from private individuals to establish new nature reserves and national parks, as other conservation organizations have to do elsewhere in the world.

Things are not so simple, however, because the division of interests between different state ministries and departments has blurred the concept of the 'single owner'. There are, it seems, many more than one — land and forests, mineral resources and waters, fish and game animals, nature reserves and national parks, all come under separate authorities. Even within a single national park, there may be as many as four 'owners'. One of the most popular expressions in the Soviet press at present is that "Many owners means no owner". This particularly applies to the administration of conservation, and, in Ziegler's view, is one of the main problems for Soviet conservationists.

Ziegler discusses in detail the issue of Lake Baikal, which caused a wave of protests throughout the Soviet Union, and also the huge project to divert the north-flowing rivers of Siberia to central Asia. During a recent visit to Britain, I was often asked to comment on this project, and the announcement that it had been cancelled was always greeted with relief. These and other recent events show how fast the environmental awareness of the government and people in the Soviet Union is growing. The Lake Baikal issue, according to Ziegler, is a "unique case of [public] participation in Soviet environmental policy". In fact, cases like this are far from uncommon, and such questions are widely debated in the press and by the public at large.

Ziegler devotes two chapters to the comprehensive and detailed discussion of Soviet environmental law and of the administrative system for the

environment. "One striking facet of Soviet environmental administration is its fragmented, uncoordinated character" he observes. This important fact is generally recognized in the Soviet Union, not least by government officials. Moves to establish a single comprehensive organization to deal with environmental problems are well advanced.

The book as originally planned concluded with a chapter on the relationship between environmental problems and Soviet foreign policy, using the particular example of Soviet-American cooperation

nature in the United States, which I myself have witnessed, and the private ownership of huge tracts of land, generate many of the same sort of problems that are found in the Soviet Union. Further, the vast size of the Soviet Union and its seemingly inexhaustible natural resources have inevitably meant slower growth in awareness of environmental issues.

What the book lacks, in my opinion, is more detail on protected areas, the care of endangered species, the functioning of the Red Data books and the participation of people in the solving of environmental problems, as these aspects demonstrate the results of policy decisions. Even for a book entirely devoted to protection policy and not its biological and social background, it would have been a considerable advantage if Ziegler had had a chance to work on the spot as an environmentalist. Nonetheless it is surprising how well he understands certain attitudes towards

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Floating forest — timber is sent downstream to Lake Baikal and on to the lumber works of Siberia.

in this field. However, Ziegler decided to write an afterword on the Chernobyl incident, and this leaves a very sour aftertaste. Naturally, once the accident had occurred, it was used for political ends (as Ziegler stresses), but it was also, and principally, of great scientific interest; and the author would have done well to remember that no country and no enterprise, regardless of political system, can possibly be accident-proof.

Ziegler cites a huge and impressive number of published sources. One should perhaps note that these titles are not always really comparable, ranging as they do from official Soviet publications to *samizdat* sources published in the West. There are, too, some highly controversial statements such as that "independent groups can work more efficiently in the USA" than in the Soviet Union. The strong consumerist attitudes towards

environmental protection in the Soviet Union, both those of the policy makers and those of ordinary citizens.

Ziegler's book has appeared at a time of rapid change in all aspects of Soviet society, and although it will be of great interest to those concerned with environmental matters it will serve more as a historical study than as an up-to-date account of Soviet policy. Environmental issues clearly now have high priority on the government's agenda, and are the subject of widespread and open debate. The effects may not be immediately apparent, particularly to the rest of the world. But we know that, for most things, in the beginning is the Word. □

Algirdas Knystautas, 232040 Vilnius 40, Zirgo 3-15, Lithuanian SSR, USSR, is a freelance nature photographer and writer. His most recent book, The Natural History of the USSR, appeared in Britain earlier this year and has just been published in the United States by McGraw-Hill.

Marine networks

David G. Ainley

Seabirds: Feeding Ecology and Role in Marine Ecosystems. Edited by J.P. Croxall. Cambridge University Press:1987. Pp.408. £30, \$59.50.

THE management of seabird populations has traditionally been the responsibility of wildlife agencies which, in general, almost entirely concentrate upon terrestrial and freshwater ecosystems. Although it is certainly appropriate that the island breeding sites of seabirds — which are in critical need of care and protection — should come under the purview of these agencies, equally important to the viability of seabird populations is the wise management of the marine food webs of which seabirds are a component. Marine ecosystems, however, usually come under the authority of fishery agencies, in which multi-species management is much talked about but is largely yet to be realized. As a result of this management dichotomy, seabird biologists have been waging an unofficial campaign for the past 15 years to try to bring marine birds to the attention of fishery managers and biologists.

The book under review is the second to appear as an outgrowth of this effort; its 'flavour' is virtually the same as that of *Marine Birds: Their Feeding Ecology and Commercial Fisheries Relationships*, edited by D.N. Nettleship *et al.*, and published in 1984 by the Canadian Wildlife Service. The two books have a number of authors in common, which doubtless in part accounts for the similar flavour, but they are actually quite complementary and do not overlap in content. *Marine Birds* contains case studies and theoretical models of the dynamics of seabird food

webs, whereas the new volume summarizes and reviews the available information on diet, foraging behaviour and interactions with marine processes. Together, the books provide an up-to-date overview of seabird foraging ecology and should interest wildlife and fishery managers, ornithologists and ecologists working on pelagic marine food webs.

In the present volume, the contribution by R.W. Furness on kleptoparasitism, its prevalence in seabirds and significance to them, is long overdue. G.L. Hunt, Jr and D.C. Schneider's account of scale-dependent processes, and how our perceptions of interactions of seabirds with their environment are a function of scale, should stimulate further research. Successful applications of this discussion are presented in chapters by Schneider *et al.* for the comparison of energy flux in shallow-water seabird communities of Bristol Bay and Georges Bank, and by R.T. Briggs and E.W. Chu for energy flux in the upwelling system off California.

The remaining contributions mainly review what and how much is eaten by certain seabird taxa (penguins, Procellariiformes, Pelecaniiformes and alcid) and faunas (Gulf of Alaska, Hawaiian Islands, California current, Humboldt and Benguela currents, and South Georgia). They contain much useful information, as do the articles on seabird flight (a component of foraging) and diving behaviour.

Information on what is known about seabird foraging ecology is now well summarized. Clearly, further progress will come mainly through studies of foraging energetics, diets outside the breeding season, diets relative to prey availability, and the relationship between foraging and population ecology. □

David G. Ainley is Director of Marine Studies, Point Reyes Bird Observatory, Stinson Beach, California 94970, USA.

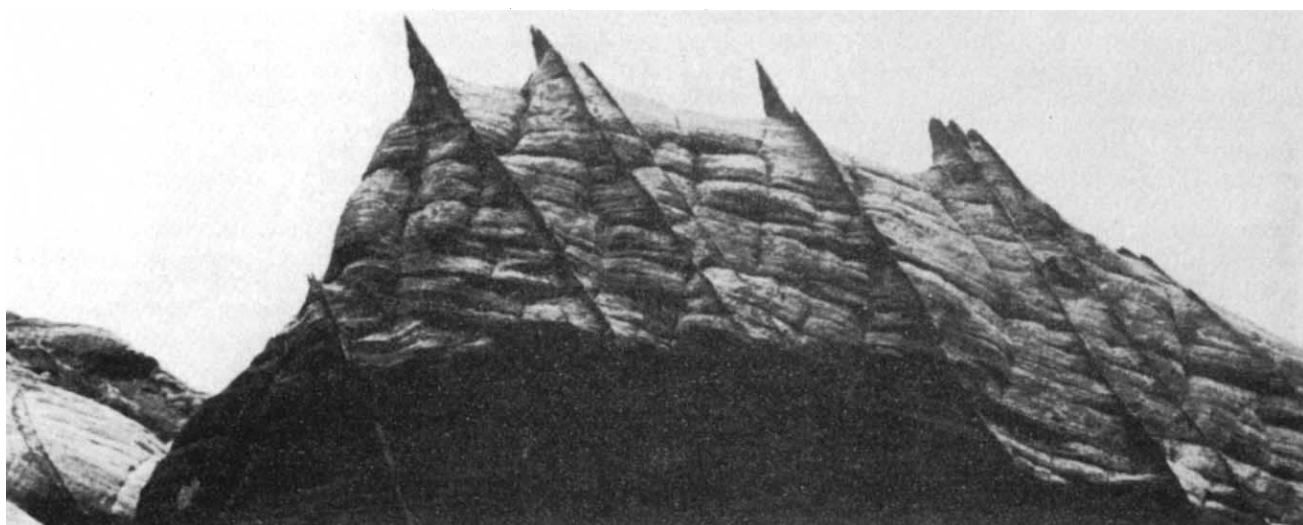
Manufacturing the mental process

John A. Campbell

Thinking Machines: The Evolution of Artificial Intelligence. By Vernon Pratt. Basil Blackwell:1987. Pp.254. £19.50, \$19.95.

ONE measure of the interest of a subject is the extent to which volunteers to write or otherwise explain its intellectual history turn up. For most subjects, this measure refers not only to interest but also to respectability. For some, including (unfortunately) artificial intelligence, the earliest volunteers have included science-fiction writers and cowboys. The pattern in these cases usually starts with a Wild West phase, in which a subject is long on public interest but short on respectability, followed by a period of inactivity on the intellectual-history front while instant applications are not forthcoming and while the specialists quietly get on with the work of consolidation and establishment of fundamental principles. If the second phase is a successful one, then the arrival of non-cowboy volunteers to describe the subject and its historical pedigree indicates that a period of combined interest and respectability has arrived with (or somewhat before) them.

The first person to attempt a thorough account of the historical development of artificial intelligence will have a difficult job because of the very wide range of material and possibilities that needs to be considered. This is an open challenge to professional historians of science. Until it is taken up convincingly, everyone is free to assemble his or her version of



Jagged edge — this formation from the Valley of Fire in Nevada shows joints with walls impregnated with silica cutting across bedded sandstones. The picture is taken from *The Techniques of Modern Structural Geology* Vol. 2: *Folds and Fractures* by John G. Ramsay and Martin I. Huber. Vol. 1 won the Geological Society of America 1985 Best Paper Award. The book is published by Academic Press and costs £17.50, \$34.50.

the history by applying do-it-yourself methods to the data. There are many possible strands on offer for this historical weaving exercise, but clearly one of the most obvious candidates for choice is the one labelled 'logic'.

Several of the non-technical books whose recent appearance has coincided with the new 'interesting-and-respectable' period for artificial intelligence are written by philosophers, and make gestures towards logic only on the way towards concentration on philosophical issues. While Vernon Pratt is also trained in philosophy his interest here is more in tracing the sources of modern ideas about representation of knowledge and mathematical abstraction, and in particular their effects on the work of three heroes of computation: Leibniz, Babbage and Turing.

In passing, the book contains many other historical morsels from which the reader can make a do-it-yourself selection. It is less of a history of either computation or the evolution of artificial intelligence (for example, the links between the past and the post-1955 highlights in artificial intelligence are rather tenuous here) than a collection of information that establishes some of the intellectual climate in which the evolution has taken place. However, it gives an excellent summary account of the historical interplay of mathematical abstraction and the aims and expressiveness of mathematical logics, especially where this interplay has led to implicit assumptions about what 'logical' knowledge is and about how it can be represented. Because of this, it is useful reading even for those who are specialists in artificial intelligence, who may learn from it to take fewer of the classical assumptions behind knowledge-based computing for granted.

It may be ironic that this book has appeared at a time when the specialists have already begun to question whether the orthodox view, that knowledge-based computing is always reducible to symbolic and logical operations, is the only sensible view to take. The recent expansion of interest in connectionist computing and neural-net architectures is one consequence of the questioning. Artificial intelligence has a habit (already demonstrated for psychology and linguistics, among other areas) of overturning or at least disturbing the standard outlooks in subjects with which it comes in contact. There is no reason to suppose that mathematical logic will be immune to this process. Some of the pre-Cartesian ideas that receive brief mentions in Pratt's first chapters may not be dead and buried yet. □

John A. Campbell is Professor of Computer Science at University College London, Gower Street, London WC1E 6BT, UK.

Gaps in progress

David Brown

Synaptic Function. Edited by Gerald M. Edelman, W. Einar Gall and W. Maxwell Cowan. Wiley:1987. Pp.789. Hbk \$149.95, £130; pbk \$39.95, £34.50.

ON receiving this book, my first thought was that it might be the ultimate text on synapses, able to reach those parts where other texts of general neuroscience could not penetrate. A closer look revealed that this is not the case: the book does not really achieve the status of a comprehensive treatise, but instead consists of a series of individual essays on various aspects of synaptic (and even non-synaptic) function. As a result, some parts of the subject are covered in detail but others are hardly touched upon.

The essays are grouped under five general headings: "Presynaptic and Postsynaptic Mechanisms"; "Neurotransmitters and Synaptic Function"; "Synapses in Networks"; "Synaptic Plasticity, Memory, and Learning"; and "Theoretical Models of Synaptic Function". These subdivisions seem somewhat arbitrary, and there are a number of outriders and omissions. For example, in the first section there are three articles on the release of chemical transmitters (Llinas, Magleby, Korn & Faber) yet none at all on their postsynaptic action; instead, the balance is made up by an article on gap junctions (Bennett & Spray), and two articles on voltage-gated channels (Catterall, Hille) which seem misplaced here. Indeed, there is surprisingly little specific discussion of postsynaptic transmitter mechanisms in the book as a whole; even in Section 2, on neurotransmitters, the topic is restricted to one article on GABA-activated channels (Olsen), though some discussion of serotonergic and nicotinic mechanisms is incorporated in articles on learning by Kandel and Changeux, respectively, in the section on synaptic plasticity.

The individual essays vary considerably both in length (from 15 to 60 pages) and in format; some are research summaries, others are more generalized and speculative expositions. The former suffer from two disadvantages: in several cases (for example, co-transmission, Na and Ca channels, GABA receptors, learning in *Aplysia*, protein phosphorylation) similar ground has been covered in other such assemblies of articles; and these contributions are squeezed by the twin pincers of rapid progress and slow publication. Thus, there are very few references later than 1985, and I'm afraid this shows. For example, the chapter on learning-related phenomena in the hippocampus antedates recent knowledge regarding the

permeability of NMDA-activated channels to Ca, which would have facilitated the author's interpretation of LTP. Even more limiting is the fact that the article on molecular properties of Na and Ca channels was apparently completed before the amino acid sequence of the Na channel was described, a problem further exacerbated by the more recent cloning of the dihydropyridine-binding component of the Ca channel. For such reasons the long-term impact of this book is more likely to be determined by the more integrative and speculative articles, such as those on probabilistic release mechanisms (Korn & Forber), dendritic function (Rall), computations (Koch & Poggio) and population rules (Finkel & Edelman).

In placing the book in context, it is worth noting that it is a publication of The Neurosciences Institute of the Neuroscience Research Program (NRP). In their original form, as published at three-monthly intervals (or thereabouts), the NRP Bulletins provided unique surveys of specific neuroscience topics which had the advantages of being focused, up-to-date and sufficiently cheap for the individual scientist to subscribe. These were progressively transmuted, firstly into the Study Programs which appeared at approximately three-year intervals, and then into the present reports of annual symposia of which this is the third, now published by Wiley and no longer particularly cheap. As a result, they have lost their unique place in neuroscience literature and have to compete with the myriad other symposial outpourings.

As such this volume is certainly worth having around because the author list is impressive and virtually every neuroscientist will find something of interest in it. Nevertheless, I personally miss the old-style NRP Bulletins; and the neuroscience world still awaits the definitive comprehensive treatise on synaptic function. □

David Brown is a Professor in the Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK, and Director of the MRC Neuropharmacology Research Group.

New editions

● **Immunochemistry in Practice** by Alan Johnstone and Robin Thorpe. Publisher is Blackwell Scientific, price is £12.50, \$29.95. For review see *Nature* 301, 182 (1983).

● **Culture of Animal Cells** by R. Ian Freshney. Publisher is Alan R. Liss, price is \$59.50. Available in the UK through Wiley, price £45.50. For review see *Nature* 307, 574 (1984).

● **Five Kingdoms: An Illustrated Guide to the Phyla of Life on Earth** by Lynn Margulis and Karlene V. Schwartz. Publisher is W.H. Freeman, price is hbk £32.50, \$35.95; pbk £21.95, \$24.95.

● **Cancer Biology** by R.W. Ruddon. Publisher is Oxford University Press, price is £25, \$29.50. For review see *Nature* 297, 714 (1982).

New developments

Benjamin G. Brackett

Experimental Approaches to Mammalian Embryonic Development. Edited by Janet Rossant and Roger A. Pedersen. Cambridge University Press: 1987. Pp.558. £47.50, \$70.

"WHY study the mammalian embryo?" Janet Rossant and Roger A. Pedersen provide three answers in their preface to this book. First, as mammals ourselves, the study of other mammals can fulfil a need to learn more of our own development. Second, work with laboratory mammals has turned out to be directly beneficial in the development of human *in vitro* fertilization as a clinical procedure. Finally, mammals represent the last embryological frontier, one that is now accessible through new techniques.

The editors' intention was to review the large body of information now available on the cellular, biochemical and molecular aspects of the early mammalian embryo, and the exciting new approaches to developmental genetics made possible by recombinant DNA techniques. The resulting volume will be of value to anyone with an interest in the mammalian embryo: advanced undergraduates,

graduate students, researchers with interests in developmental and molecular biology, and applied scientists with an eye on future applications. The reviews are well written and detailed, reflecting the expertise of each of the 21 authors, with literature cited up to early 1986.

The book is divided into three general sections: cellular aspects, including studies of potency, allocation, differentiation, and fate in pre-implantation and early post-implantation embryos; molecular aspects, including gene expression during gametogenesis and early development, physiological aspects, cell surface and cytoskeletal differentiation, and X-chromosome inactivation; and new approaches towards a genetic understanding of development, including nuclear transfer, analysis of genetic mutations, and introduction of new genetic material into the embryo via stem cell lines and production of transgenic mice.

One cannot peruse this volume without being struck by the overwhelming contribution of research on the laboratory mouse to our knowledge of early development. For me, however, those authors who depart from the mouse model

represented an occasional and refreshing interlude. Thus the caution expressed by Virginia E. Papaioannou and Karl M. Ebert — "Although the broad outline of cell lineage relationships can be extrapolated using the mouse as a model, the details can be ascertained only by experimental studies on representative species" — seems particularly appropriate, not only in that context but to other aspects of the subject discussed in the book. Many of the approaches pioneered by work with the laboratory mouse point the way for studies in other mammals where important agricultural or medical applications are becoming increasingly obvious. The editors and authors are to be commended for their service in indicating such routes for future research. The objective of the editors was to summarize advances in mammalian embryology during the past decade, and they have succeeded. This book should indeed be a valuable addition to the library of "any serious student of the mammalian embryo". □

Benjamin G. Brackett is a Professor in the Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, USA.

By any other name

P.V.E. McClintock

Dictionary of Effects and Phenomena in Physics. By Joachim Schubert. VCH: 1987. Pp.140. DM 54, £27.45.

GIVEN the maturity of physics as a science and its continuing rate of expansion (almost two metres of new shelf-space per annum per library being needed for *Physical Review* alone), the relatively modest dimensions of Joachim Schubert's *Dictionary of Effects and Phenomena in Physics* come as something of a surprise. The main alphabetical listing runs to a mere 88 (smallish) pages, so that one is bound to question whether the subject has been covered: is it really all there?

The book is a new edition, in English, of an earlier German version. Its stated aim is to provide an overview for a broad readership of some 400 physical effects and phenomena, including historical notes about the discoveries and their discoverers together with references to the pertinent literature. The greater part of the volume consists of the dictionary itself, providing a succinct description of each effect or phenomenon and references to the original papers. There is also a series of 55 tables, dividing the phenomena into coherent groups, such as "low temperatures" or "magneto-optics" or "synergetic effects". A detailed chronology is provided, from "1756 Leidenfrost phenomenon" up to "1984 Jaccarino-Peter effect" and a select bibliography of

secondary sources (mostly books).

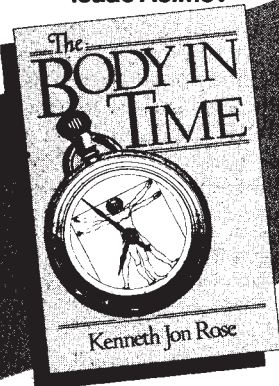
Readers should be warned that one can sometimes fail to find entries relating to important phenomena. Usually, such entries are indeed included, but under unexpected headings and without there necessarily being cross-references from the more conventional terms. For example: "Quantum Hall effect" appears as "Von Klitzing effect", a perfectly reasonable appellation though not yet in common usage outside Germany; "Superfluidity" appears rather mysteriously under "Onnes effects", and it need imply no disrespect to the father of low-temperature physics to point out that the phenomenon in question was in fact not identified as such until a decade after Onnes' death in 1926; the "Aharonov-Bohm effect" is listed (not unreasonably but, in my experience, slightly unusually) as the "Bohm-Aharonov effect"; and so on. Thus, before abandoning the quest for any particular item, it is well worth considering alternatives and consulting the relevant table to see whether it has been included under a different name.

Although not quite the whole of physics is represented in the *Dictionary*, a very sizeable fraction of it certainly seems to be and the author is to be congratulated on encompassing so huge a mass of material in so small a space. The result is a handy reference work which should prove useful to many scientists and, in particular, to physicists straying outside their own particular specialisms. □

P.V.E. McClintock is a Reader in the Department of Physics, University of Lancaster, Lancaster LA1 4YB, UK.

"Fascinating..."

—Isaac Asimov



A surprising exploration of the ways our inner "organic clock" regulates everything in our bodies—from the life span of blood cells to the aging process itself.

"...one of the few writers who can blend science, humanity, and subtle humor into a highly readable and completely enjoyable book."

—Ben Bova

THE BODY IN TIME
Kenneth Jon Rose \$19.95

WILEY

Available at your bookstore

Microwaves: the risks of risk research

Kenneth R. Foster and William F. Pickard

After 40 years of work, the literature on the biological effects of exposure to low-level microwave energy remains shot through with ambiguity. Guidelines need to be established for halting risk research.

MICROWAVE radiation is one of the most thoroughly studied of all potential environmental hazards. Many of the thousands of papers that have been published on the subject are concerned with heating of tissue, which, when excessive, is clearly hazardous. Yet, fundamental questions are still being raised about the very existence of hazards associated with low levels of exposure, and significant public concern remains¹.

In this article we sketch the development of the most influential American standard for microwave exposure, and describe the history of three reported biological effects of low-level microwave energy that have played some role in the public controversy. We do not argue that low-level microwave energy is hazardous or take issue with these standards; rather, the problems evident over four decades of research make a useful case history of the difficulties involved in demonstrating the safety of environmental agents of all sorts.

Standards

The first American exposure standard for microwave energy can be traced to the recommendation by Herman Schwan in 1953 that exposure to microwave radiation should not exceed an incident power density of 100 W m^{-2} . Schwan had calculated that such exposure (equivalent to one-eighth the energy flux of bright sunlight) would lead to additional heat loads below those generated by the body itself and to maximum increases in tissue temperature of 1°C . Schwan's recommendation was eventually embodied in Standard C95.1-1966 of the United States of America Standards Institute, later renamed American National Standards Institute (ANSI)². Substantial changes were adopted in 1982 in response to engineering studies that showed an increase in whole-body absorption near 70–100 MHz due to the antenna-like properties of the body³. The goal of the 1982 version was to limit the whole-body absorbed power to 0.4 W kg^{-1} , corresponding to about one-half the basal metabolic rate of the human body.

Although relying on engineering studies of dosimetry, the ANSI standards were approved only after critical reviews of the biological literature failed to uncover convincing evidence of harmful effects in animals exposed at levels well

above the proposed limits. This standard, although voluntary, has been the most influential of several that have been developed in the United States; similar guidelines have been adopted by other Western countries⁴.

But what constitutes evidence for a hazard? There is now an enormous literature on the biological effects of microwave energy. Many effects have been reported, some at levels that would be allowed by the ANSI standard, some of which might be considered harmful. Many of these reports are too brief to evaluate, while others have obvious technical flaws. Much of the controversy surrounding microwave energy has arisen from different interpretations of them.

The following descriptions are of three effects of electromagnetic fields that have been reported to occur under exposure conditions allowed by the 1982 ANSI standard. These effects have been chosen because they were extensively followed up, and played some role in the public controversy about possible health hazards of low-level microwave energy.

Microwave auditory effect. This effect was reported in 1947 by an observer who, standing in the beam of a radar antenna, reported hearing 'clicks' at the repetition rate of the microwave pulses⁵. To elicit this sensation, the head must be exposed to pulsed microwave energy of high peak intensity (above 2.5 kW m^{-2}). However the individual pulses need be only a few microseconds in duration, so the average power level of exposure might be well within ANSI standards.

Because the energy contained in each pulse was sufficient to heat the brain by only a few microdegrees, direct heating effects could be assumed to be insignificant. Initial efforts to detect electrophysiological sequelae (cochlear microphonics) of ordinary hearing were not successful. Other experiments in which the microwave frequency (and hence pattern of absorption within the head) was varied suggested that the locus of the sensations was in the cortex of the brain⁶. The possibility of direct neural stimulation by microwave energy thus arose.

In 1974 it was proposed that the 'clicks' are elicited by acoustic transients arising from thermal expansion in tissues subject to rapid microwave heating⁷. The initial evidence consisted of simple calculations

and measurements on water-filled containers which showed that the acoustic transients generated by the microwave pulses should be of audible intensity. Investigators in several institutions soon confirmed and extended this hypothesis in various ways. Eventually cochlear microphonics were recorded, and the 'clicks' were shown to be an ordinary hearing phenomenon.

Explanation

In retrospect, the difficulties in explaining this effect are easy to understand. The initial failure to record cochlear microphonics arose from their extremely small amplitude because the acoustic stimulus was close to the threshold for perception. The temperature rise in the brain produced by a microwave pulse is physiologically insignificant, but the rapid rate of heating is sufficient to produce audible vibrations within the head. Previously unexplained behavioural responses of animals to pulsed microwave energy could now be tested against the hypothesis that they were elicited by microwave-induced auditory cues⁸. The microwave auditory effect is not now considered a hazard — but until a compelling explanation was provided, no one could tell for sure.

Heart-rate effects. Reports that high-frequency electromagnetic radiation can affect the heart-rate go back to the work of Erich Pflumm in the early 1930s (ref. 9), who reported a slowing of the beat of isolated frog hearts when exposed to 75 MHz fields of uncertain power level. On the whole this result was not corroborated by subsequent work; more commonly investigators found an increase in heart rate with exposure, which they attributed to temperature increases in the tissue. The flurry of activity on this effect had died out by the onset of the Second World War¹⁰.

Without reference to this earlier controversy, one group reported in 1968 that exposure of isolated frog hearts to bursts of 1.425 GHz energy synchronized with the electrocardiogram led to arrhythmias, sometimes even to cardiac arrest¹¹. The time-averaged incident power density was approximately 0.006 W m^{-2} , more than four orders of magnitude below the ANSI exposure standard in effect before 1982. In part because of the brief description of the experiment, no close replication has been carried out; less than perfect

attempts failed to detect such changes^{12,13}. Other workers, using continuous-wave energy at 960 MHz and absorbed power levels of 1–10 W kg⁻¹ (much higher than those intended by the 1982 ANSI C95.1 standard), reported a slowing in the rate of isolated turtle and rat hearts, which was opposite in direction from that expected from simple heating^{14–16}. Subsequent studies, employing widely different techniques and exposure conditions, and various heart preparations, have found^{17,18} or not found^{19–21} changes in the beat rate attributed to an effect of the irradiation. One of the original investigators has recently reported a small increase in the rate of beating of hearts in pithed frogs irradiated with pulsed 1.25 GHz fields of low average intensity (0.03 W m⁻²) synchronized with the electrocardiogram²².

Contentiousness

There the matter stands. Which of the reported effects were due to experimental error (electrode artefacts, for example²⁰) and which of the negative follow-up studies were inadequate to have confirmed an earlier positive finding²² is still, in some quarters, a contentious issue.

Blood–brain barrier effects. The blood–brain barrier is a physiologically and anatomically complex system in vertebrates that protects sensitive brain tissues from ordinary variations in the composition of the blood, while allowing transport of nutrients into the brain. The first report that exposure to microwave fields was associated with a change in the barrier permeability appeared in 1972 (ref. 23). The debate began, however, with the report in 1975 that irradiation of anaesthetized rats by pulsed 1.2 GHz microwaves at time-average power densities as low as 2 W m⁻² could lead to an increased uptake by the brain of a fluorescent dye that had been previously injected into the blood stream²⁴. Similar effects with a different tracer and different experimental techniques were reported shortly thereafter²⁵ in rats exposed to 1.3 GHz radiation of intensity below 30 W m⁻². The significance of these initial findings was difficult to judge, but clearly any such direct effect on the cerebrovascular permeability, and thus indirectly on the central nervous system, would be cause for concern. The existence of an effect of low-level irradiation could, around 1977, have been considered independently confirmed.

Other investigators were unable to corroborate these results. Merritt *et al.*²⁶ could not confirm two of the earlier studies, but Frey later argued that their data did show an effect of irradiation²⁷. (This effect, however, was correlated with changes in the body temperatures of the exposed animals, which were anaesthetized and thus had impaired thermoregulation.) Preston *et al.*²⁸ in 1979 failed

to corroborate the changes in brain uptake index (BUI) reported earlier²³. They concluded that low-level microwaves did not increase the barrier permeability, and that the effect on the BUI was an artefact of changes in blood flow. Other investigators reported a change in the permeability of the blood–brain barrier but only after high-level exposure that raised the brain temperature to 40–43°C (ref. 29).

At least 15 groups worked on the problem in the 13 years following the original reports of the effect. Few investigators would now claim that the blood–brain barrier is greatly affected by microwave irradiation at levels anywhere near those allowed by past or present ANSI exposure standards.

What happened? Measurements of the barrier permeability are easily confounded by extraneous factors. One critical review³⁰ describes two thermally induced changes in particular that might have misled the early investigators: changes in the rate of excretion of the tracer, and changes in brain blood flow. Subsequent studies were less susceptible to such artefacts. One of the investigators who first reported an effect of microwave exposure at levels below 100 W m⁻² has since carried out³¹ a study employing an improved technique, with 200 times the sensitivity of his earlier measurements, and has found no changes in the permeability of the blood–brain barrier over the range of power levels employed (0–400 W m⁻²). Thus, the earlier observations in part could not be confirmed and in part might have been artefacts.

Many other reported effects — perhaps hundreds of them — remain to be followed up. There is the same confusion over other parts of the electromagnetic spectrum. One review lists 127 effects that have been claimed to arise from 50–60 Hz electric fields, at field strengths ranging from near the dielectric breakdown strength of air to ~1 V m⁻¹ (ref. 32). Of these, approximately a quarter have been independently confirmed, a third apparently negated, and the rest have not been investigated in enough detail for either conclusion to be drawn. (Most of the confirmed effects occur at high-field levels and are associated with obvious physical phenomena, corona for example.) Some 50 effects have been claimed to be associated with exposure to 50–60 Hz magnetic fields, of which barely one-tenth have been independently confirmed. The effects described above illustrate how easy it is to collect evidence which neither demonstrates a hazard nor provides assurance that no hazard is present.

The problem is not (as is frequently asserted) that too little is known about the biological effects of microwave energy. Rather, endless exploratory research has yielded many observations the importance of which cannot be reliably judged,

and which frequently cannot be independently confirmed. Because an 'effect' might be reported by an exploratory study that took weeks to carry out, whereas it might require years to confirm the existence of the phenomenon and to judge its significance, there can be no end to controversy save by exhaustion or by taking the conscious decision to leave some questions unanswered. The state of the microwave bioeffects literature is a lesson for those intending to study the many other environmental agents for which potential hazards have yet to be explored.

Granted, society must search for hazards of its technologies. But how to cope with the scientific noise that these studies produce? Such searches for hazards can go on too long, and guidelines for ending them must be established. □

1. Steneck, N.H. *The Microwave Debate* (MIT Press, Cambridge, Massachusetts, 1984).
2. *Safety Level of Electromagnetic Radiation with Respect to Personnel* USAS Standard C95.1–1966 (United States of America Standards Institute, 1966).
3. *Safety Levels with Respect to Human Exposure to Radio Frequency Electromagnetic Fields, 300 kHz to 100GHz* ANSI Standard C95.1–1982 (American National Standards Institute, 1982).
4. Clemmensen, J. *Nonionizing Radiation: A Case for Federal Standards?* (San Francisco Press, 1984).
5. Chou, C.-K., Guy, A.W. & Galambos, R.J. *Acoust. Soc. Am.* **71**, 1,321–1,334 (1982).
6. Frey, A.H. *J. Appl. Physiol.* **23**, 984–988 (1967).
7. Foster, K.R. & Finch, E.D. *Science* **185**, 256–258 (1974).
8. Chou, C.-K., Guy, A.W. & Galambos, R.J. *Acoust. Soc. Am.* **71**, 1,321–1,334 (1982).
9. Pfommer, E. *Arch. Klin. Chir.* **166**, 251–305 (1931).
10. Osborne, S.L. & Holmquest, H.J. *Technic of Electrotherapy* (C.C. Thomas, Springfield, Illinois, 1944).
11. Frey, A.H. & Seifert, E. *Life Sci.* **7**, 505–512 (1968).
12. Clapman, R.M. & Cain, C.A. *J. Microwave Power* **10**, 411–419 (1975).
13. Liu, L.-M., Rosenbaum, F.J. & Pickard, W.F. *J. Microwave Power* **11**, 225–232 (1976).
14. Lords, J.L., Durney, C.H., Borg, A.M. & Tinney, C.E. *IEEE Trans. Microwave Theory Tech.* **MTT-21**, 834–836 (1973).
15. Tinney, C.E., Lords, J.L. & Durney, C.H. *IEEE Trans. Microwave Theory Tech.* **MTT-24**, 18–24 (1976).
16. Olsen, R.G., Lords, J.L. & Durney, C.H. *Ann. Biomed. Eng.* **5**, 395–409 (1977).
17. Chalker, R., Wachtel, H. & Barnes, F. *Abst. 3rd Ann. Conf. Bioelectromagnet. Soc.* **80** (1981).
18. Caddemi, A., Tamburello, C.C., Zanforlin, L. & Torregrossa, M.V. *Bioelectromagnetics* **7**, 359–367 (1986).
19. Yee, K.-C., Chou, C.-K. & Guy, A.W. *Bioelectromagnetics* **5**, 263–270 (1984).
20. Yee, K.-C., Chou, C.-K. & Guy, A.W. *J. Microw. Power* **21**, 159–165 (1986).
21. Watkinson, W.P. & Gordon, C.J. *Am. J. Physiol.* **250**, H320–H324 (1986).
22. Frey, A.H. & Eichert, E.S. *J. Bioelectricity* **5**, 201–210 (1986).
23. Polyashchuk, L. *Dokl. Akad. Nauk. Ukrain.* **8**, 754–758 (1972).
24. Frey, A.H., Feld, S.R. & Frey, B. *Ann. N.Y. Acad. Sci.* **247**, 433–438 (1975).
25. Oscar, K.J. & Hawkins, D. *Brain Res.* **125**, 281–293 (1977).
26. Merritt, J.H., Chamness, A.F. & Allen, S.J. *Rad. Environ. Biophys.* **15**, 367–377 (1978).
27. Frey, A.H. *Bioelectromagnetics Soc. Newsl.* **1**, 4 (1980).
28. Preston, E., Vavasour, E.J. & Assenheim, H.M. *Brain Res.* **174**, 109–117 (1979).
29. Lin, J.C. & Lin, M.F. *Rad. Res.* **89**, 77–87 (1982).
30. Williams, W.M. *et al. IEEE Trans. Microwave Theory Tech.* **MTT-32**, 808–817 (1984).
31. Gruenau, S.P., Oscar, K.J., Folker, M.T. & Rapoport, S.I. *Exp. Neur.* **75**, 299–307 (1982).
32. Carstensen, E.L. *Biological Effects of Transmission Line Fields* (Elsevier, New York, 1987).

Kenneth R. Foster is in the Department of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA, and William F. Pickard is in the Department of Electrical Engineering, Washington University, St Louis, Missouri 63130, USA.

The Western Equatorial Pacific Ocean Circulation Study

Eric Lindstrom*, Roger Lukas†, Rana Fine‡, Eric Firing†, Stuart Godfrey*, Gary Meyers* & Mizuki Tsuchiya§

*CSIRO Division of Oceanography, GPO Box 1538, Hobart, Tasmania 7001, Australia

†Joint Institute for Marine and Atmospheric Research, University of Hawaii, 1000 Pope Road, Honolulu, Hawaii 96822, USA

‡Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, Florida 33149, USA

§Institute of Marine Resources, A-030, Scripps Institution of Oceanography, La Jolla, California 92093, USA

A recent set of oceanographic measurements in the western equatorial Pacific has revealed the existence of previously undescribed major ocean currents and upper-ocean mixed-layer structure. The measurements also confirm a 20-year-old hypothesis on the water-mass origins of the Equatorial Undercurrent in the Pacific Ocean.

OCEANOGRAPHIC research on the El Niño Southern Oscillation (ENSO) has, until recently, focused on phenomena occurring in the eastern and central Pacific Ocean. Large interannual sea surface temperature anomalies, wind events and ecosystem disruption in these regions have been related to ENSO events. However, the early stages of ENSO events may originate in the western equatorial Pacific¹ and recent studies have focused on the regional heat storage as a factor in the climate system^{2,3}. Understanding heat storage requires information on ocean currents and water properties, but the general circulation of the low-latitude western Pacific Ocean is poorly known compared with that of the Atlantic and Indian Oceans. The relatively complex topography of the western boundary, including openings through the Indonesian archipelago, must strongly influence this circulation. The Western Equatorial Pacific Ocean Circulation Study (WEPOCS) was conceived to study these problems. A premise in the experimental design was that further knowledge of the seasonal cycle in the far western Pacific would increase our understanding of the early stages of ENSO events. It is based on the observed phase-locking of ENSO phenomena with the seasonal cycle^{4,5}. Furthermore, it was also thought that poorly understood aspects of the west Pacific circulation, such as the response of the ocean to the monsoonal wind forcing, and the structure of the upper ocean and mixed layer, play a critical role in ENSO events.

Two expeditions were conducted, the first in June–August 1985 (WEPOCS I) and the second in January–February 1986 (WEPOCS II), by investigators from Australia and the United States. The cruises were timed to coincide with the peaks of the south-east and north-west monsoons. Work was concentrated in the region north and east of Papua New Guinea (Fig. 2). The currents at three sites and sea level at six sites were also measured over the 6-month period between expeditions. Complementary measurements were made by the Australian research vessel *Franklin* (WEPOCS I & II) and the United States research vessels *Thomas G. Thompson* (WEPOCS I) and *Moana Wave* (WEPOCS II).

The main observational components were a grid of conductivity–temperature–depth (CTD) stations (including discrete sampling for O₂, NO₃, PO₄, SiO₂, ³He and ³H) extending from 143° E to 155° E and from 8° S to 5° N, current meter moorings in Vitiaz Strait, St Georges Channel, and the Equator at 150° E, full depth velocity profiling stations from 1° S to 2° N along 150° E, pressure measurements in the Solomon Strait and Vitiaz Strait, and acoustic Doppler current profiler (ADCP) measurements by all three WEPOCS research vessels. Special attention was paid to sampling of western boundary currents along the coast of Papua New Guinea and to the collection of deep profiles in previously data-poor areas.

This article will provide an overview and draw attention to

some of the major scientific results of WEPOCS. A comprehensive set of detailed analyses arising out of the WEPOCS work will further advance the description of the physical oceanography of the western equatorial Pacific Ocean.

Undercurrent origins

The WEPOCS programme tested a hypothesis of Tsuchiya⁶ that the Coral Sea is the primary source for the high-salinity waters of the Pacific Equatorial Undercurrent in the western Pacific. Tsuchiya suggested that they came from the tongue of high-salinity water found along the north coast of Papua New Guinea (PNG) in the Bismarck Sea; this water in turn must originate from the south, via the Solomon and Coral Seas, which would imply that there is a large net flux of water northward through Vitiaz Strait. The results of the WEPOCS current meter measurements in Vitiaz Strait and water property analysis support the hypothesis.

Direct current measurements in Vitiaz Strait indicated north-westward flow in the 200–700 m depth range throughout the six

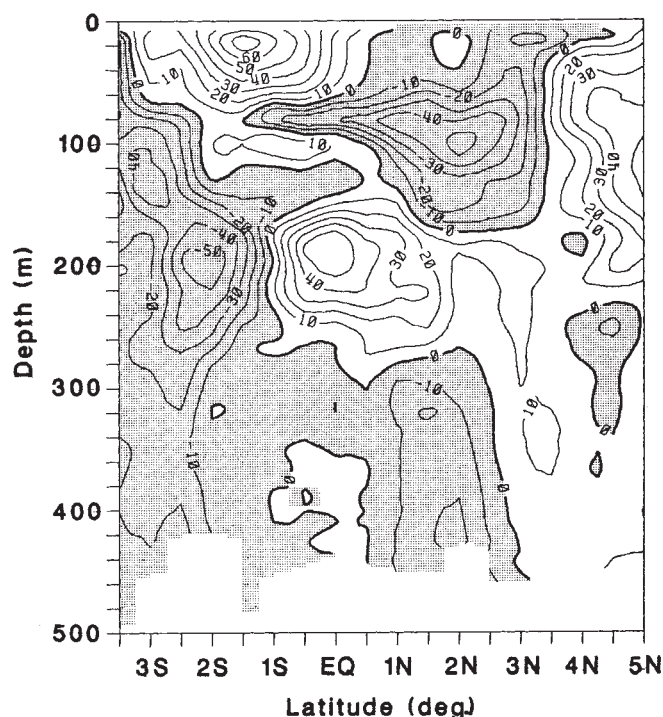


Fig. 1 Section of zonal velocity (in cm s⁻¹) along 143° E from the Papua New Guinea Coast to 5° N. Shaded areas indicate westward flow.

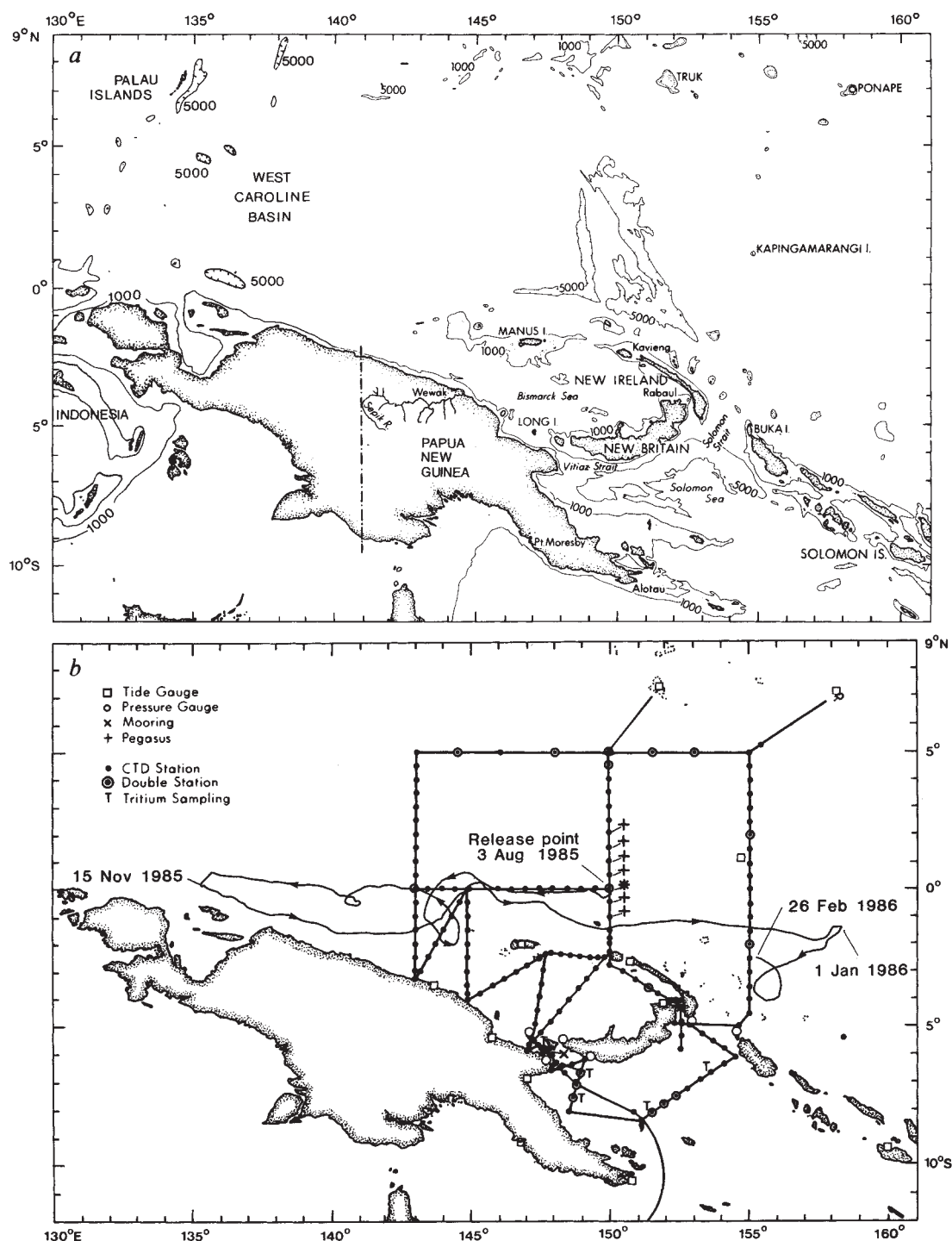


Fig. 2 *a*, Geographic features of the WEPOCS region. *b*, Cruise track and sampling scheme for the second WEPOCS expedition (January-February 1986). The track of a drifting buoy launched on 3 August 1985 is superimposed. The buoy grounded in the Nuguria Island group, east of New Ireland, on 26 February 1986.

months of observation, despite a complete reversal of the wind forcing over that time. Average absolute transport through the strait, as estimated from the mooring (current meters at average depths of 350, 450 and 550 m) and ADCP sections (nine traverses of the strait) is $8 \times 10^6 \text{ m}^3 \text{ s}^{-1}$. The continuation of sub-surface northwestward flow from Vitiaz Strait along the coast of PNG is illustrated in a velocity section from WEPOCS II along 143° E (Fig. 1). A 50 cm s^{-1} core in zonal current speed is centred at 200 m adjacent to the coast at 2° S. This sub-surface equatorward velocity maximum we named the New Guinea Coastal Undercurrent. The continuity of this circulation feature is illustrated in Fig. 3b, which shows all ADCP current estimates averaged over the range 146–210 m. At 143° E absolute transport in both New Guinea Coastal Undercurrent and Equatorial Undercurrent is $7 \times 10^6 \text{ m}^3 \text{ s}^{-1}$ (including only those regions with speeds

greater than 40 cm s^{-1}). It is not yet known how much of the transport of this undercurrent is returned to the east in the Equatorial Undercurrent and how much continues to the west, perhaps participating in the throughflow from the Pacific to the Indian Ocean via the Indonesian archipelago.

Water-property maps from the second WEPOCS expedition show that high-salinity, low-nutrient water is found in a core centred at $\sim 300 \text{ cl tonne}^{-1}$ thermocline anomaly between 150 and 220 m on the western side of the Solomon Sea (Fig. 3). This high-salinity core can be traced through Vitiaz Strait and along the northern New Guinea coast, where it coincides with the sub-surface equatorward velocity maximum of the New Guinea Coastal Undercurrent. The depth of this surface (Fig. 3a) varies from 150 m to 220 m. Salinity on the surface (Fig. 3c) exceeds 35.6‰ in the Solomon Sea and through Vitiaz Strait northwest-

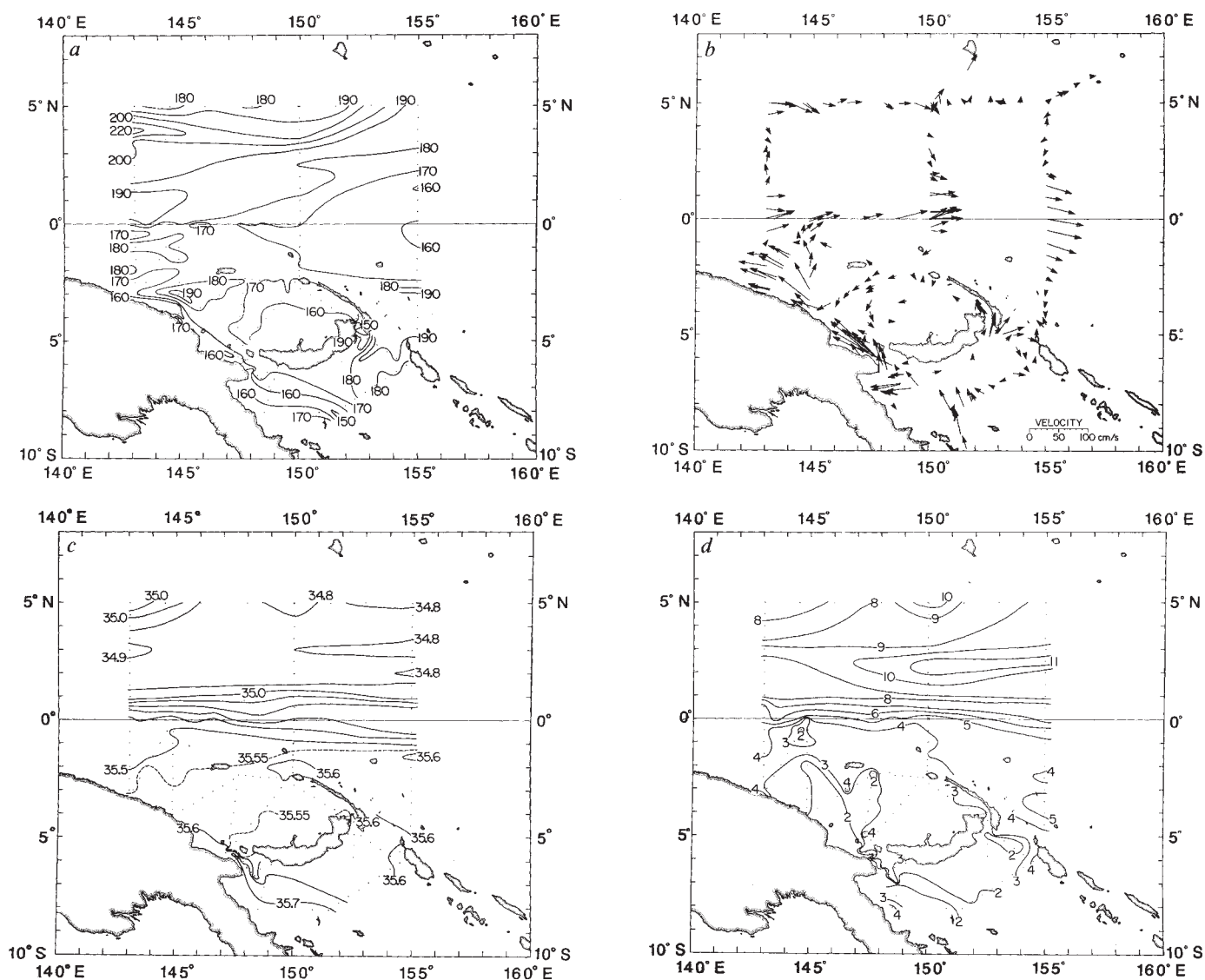


Fig. 3 *a*, Pressure in decibars of the 300 cl tonne⁻¹ thermosteric anomaly surface during WEPOCS II. *b*, Current vector map for the depth range 146–210 m obtained by acoustic Doppler current profilers on board the RV *Franklin* and RV *Moana Wave* during January–February 1986. *c*, Salinity (‰) on the 300 cl tonne⁻¹ surface during WEPOCS II. *d*, Silicate concentration (μmol l⁻¹) on the 300 cl tonne⁻¹ surface during WEPOCS II.

ward along the Papua New Guinea coast. The silicate concentration (Fig. 3*d*) is low in the Solomon Sea (except where rivers enter the sea) and patterns of silicate concentration clearly exhibit the intrusion of Solomon Sea waters through Vitiaz Strait into the western Bismarck Sea. Salinities exceeding 35.6‰ are also found to the north of New Ireland. This high-salinity tongue does not extend much beyond 150° E in any of the WEPOCS data. This observation, in conjunction with the similarity of transport estimates of the New Guinea Coastal Undercurrent and Equatorial Undercurrent at 143° E, suggests that the major source of high-salinity water in the Equatorial Undercurrent is that derived from the Vitiaz Strait throughflow. Waters of similar properties continue to be entrained in the undercurrent as it passes north of New Ireland. Water-property maps from the first WEPOCS expedition (not shown) also illustrate the above points.

The high-salinity core of the New Guinea Coastal Undercurrent in Vitiaz Strait is also coincident with a relatively high-tritium core, which when decay-corrected is similar to tritium values off the east Australian coast⁷. These data are consistent with the idea of the equatorward-flowing water originating in the vicinity of the Great Barrier Reef Coastal Undercurrent^{8,9}

in the western Coral Sea. The two currents may be connected through an eastward-flowing undercurrent south of Papua New Guinea and northwestward-flowing undercurrent at the western boundary of the Solomon Sea. Observations indicating the existence of these undercurrents were made during the Transports of Equatorial Waters Expedition in July 1987.

Monsoon forcing

The average wind stress fields for July 1985 and January 1986 are shown in Fig. 4. Southeasterly wind forcing is predominant over the WEPOCS area in July; northwesterly forcing predominates along the equator in January. A current meter mooring was placed in 5,250-m water depth on the Equator at 150° E to measure the upper-ocean response to this change in wind forcing and the possible response of the Equatorial Undercurrent. The mooring's sea-surface toroid buoy was instrumented with an anemometer, along with air and sea temperature sensors. Below the surface buoy, current meters were placed at 15, 50, 125, 200, 225 and 350 m depth. The low-pass-filtered (running weekly average) time series of zonal current at all six levels are shown in Fig. 5. Also shown is the low-pass-filtered zonal wind component. The wind time series is obtained from the gridded

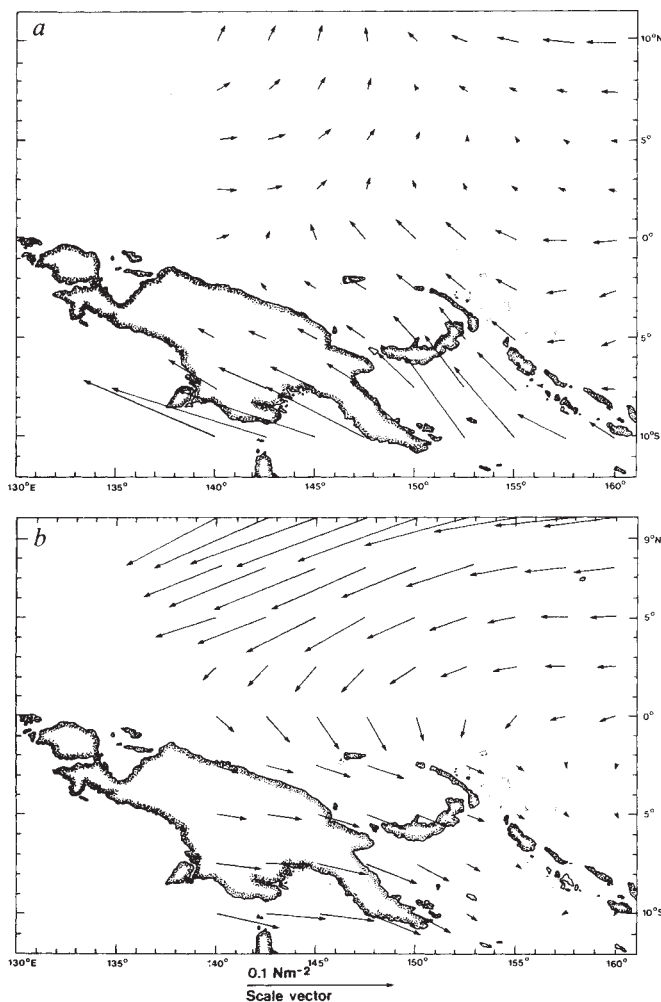


Fig. 4 Wind stress vectors in N m^{-2} for *a*, July 1985 and *b*, January 1986.

analysis values (see Fig. 4) at the mooring site. The mooring wind record ceased after 10 weeks, owing to a collision with a ship. Over the 10-week record the mooring wind measurements and 950-hPa (1 hPa = 1 mbar) gridded analysis are highly correlated ($r=0.86$) and statistically similar, so the longer gridded analysis is shown. The early part of these time series shows the vertical structure of currents under southeasterly wind forcing. During this time the zonal flow in the upper 50 m is toward the west in the South Equatorial Current, with a peak speed of $\sim 50 \text{ cm s}^{-1}$. Below the South Equatorial Current the eastward flow of the Equatorial Undercurrent is observed at 200 m and 225 m depth, with a peak speed of 35 cm s^{-1} . The gradual reversal to westward flow at 125 m is interpreted as a deepening of the South Equatorial Current.

Although bursts of westerly wind occurred from time to time, the onset of nearly continuous westerly winds associated with the monsoon transition clearly occurred in early November 1985. During this period we documented two unusual features of the upper-ocean currents. During the westerly wind forcing, the strongest zonal flows in the Equatorial Undercurrent were observed, with peak speeds of 60 cm s^{-1} found during late December 1985. During both WEPOCS cruises the pressure gradient along the equator (143°E to 150°E) at the level of the undercurrent, as indicated by the 200/1,000 dbar dynamic height, was toward the east ($3.2 \times 10^{-7} \text{ m s}^{-2}$ in July 1985 and $0.4 \times 10^{-7} \text{ m s}^{-2}$ in January 1986). This suggests that a westward pressure gradient is not necessary to drive the undercurrent in the far west Pacific and that its existence must be the result of the continuity requirements. The other important observation

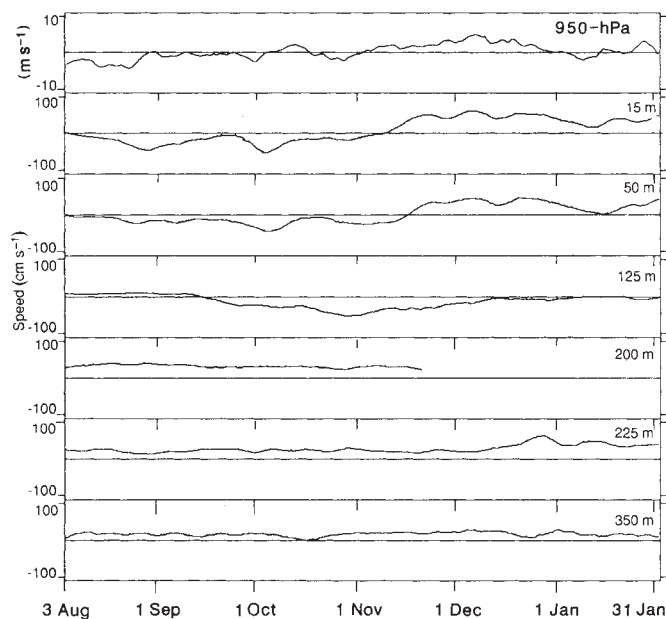


Fig. 5 Time series of zonal currents in cm s^{-1} at 15, 50, 125, 200, 225 and 350 m at 0° , 150°E from 3 August 1985 through 31 January 1986. Eastward speed is positive. Also shown is the zonal wind time series in m s^{-1} for the same period obtained from the 12-hourly gridded 950-hPa analysis of the Australian Bureau of Meteorology Research Center.

was that, within a week of the onset of westerlies, the zonal flow at the surface reverses. The surface expression of the South Equatorial Current was replaced by an eastward current distinct from the Equatorial Undercurrent, which we named the Pacific Equatorial Monsoon Jet. The jet is seen first in the 15-m record and later at 50 m. Eastward flow does not extend downward as far as 125 m, where a weak sub-surface expression of the South Equatorial Current remains until the end of the record. A similar jet-like current has been observed and described in the Indian Ocean¹⁰⁻¹².

Additional information on the upper-ocean response to wind forcing was obtained from a satellite-tracked drifting buoy launched on 3 August 1985 at the current meter mooring site on the Equator. The track of the drifter, drogued at 15 m depth, is shown in Fig. 2. It remained close to the Equator for most of its lifetime. This is unusual when compared with drifter results in the eastern Pacific, where divergence from the Equator is frequently observed¹³. Between the beginning of August and early November the drifter moved westward in the South Equatorial Current. In early November it decelerated and began moving eastward after the onset of the monsoon. Eastward flow along the Equator east of 145°E , as exhibited in both the buoy and current meter records, is not shown in previous surface current charts of the area^{14,15}. A comparison of the buoy track and current meter record at 15 m depth at 150°E shows a remarkably similar course of events, despite their large horizontal separation. The monsoon transition at 135°E , as interpreted by the buoy track, occurs simultaneously with the current reversal at 150°E (Fig. 5). Correlation analysis shows no evidence of propagation of signals between the buoy location and the mooring site, suggesting that the current reversal is locally forced by the large-scale wind pattern. From the buoy/current meter comparison we conclude that the upper-ocean reaction to large-scale wind forcing in the western equatorial Pacific is as a thin slab with a large horizontal extent and relatively short timescale.

Upper-ocean mixed layer

Prior to WEPOCS, it was thought that the mixed layer in the western equatorial Pacific was $\sim 100 \text{ m}$ deep^{16,17}. This was based

mainly on temperature sections along the Equator^{18,19}. WEPOCS investigators were surprised to find that the mixed-layer depth was, on average, considerably shallower. Using a vertical density gradient criterion of 0.01 kg m^{-3} per metre of depth, the mean depth from 242 WEPOCS CTD stations²⁰ was 29 m, with a standard deviation of 26 m. The greatest depth observed was 106 m, not long after a burst of westerly winds of up to 15 m s^{-1} . A shallower mixed layer is consistent with the light mean winds found in the western equatorial Pacific and considerable downward buoyancy flux.

On both WEPOCS cruises, a relatively deep, nearly-isothermal layer was often found to contain a shallow isohaline layer with a strong halocline at its bottom. This observation is important, because if there is no vertical temperature gradient at the base of the actively mixing layer (defined by the top of the halocline), then there can be no heat flux through the mixed layer into the thermocline. To explain this phenomenon we may invoke either vertical or horizontal processes. Either intermittently strong wind forcing is sufficient to create a deep mixed layer which re-stratifies owing to the fresh-water flux, or the mismatch in depth of isohaline and isothermal layers is a reflection of a similar discrepancy in the zonal scales of fresh-water and heat budgets along the Equator in the Pacific. The latter hypothesis, assuming relatively weak zonal temperature gradients, suggests that saltier mixed-layer waters formed in the central equatorial Pacific are subducted below the fresher (and therefore lighter) mixed-layer waters formed locally in the western equatorial Pacific²⁰.

Received 4 June; accepted 7 October 1987.

1. Barnett, T. P. *J. phys. Oceanogr.* **7**, 633-647 (1977).
2. Nicholls, N. *Nature* **307**, 576-577 (1983).
3. Meyers, G., Donguy, J. R. & Reed, R. K. *Nature* **323**, 523-526 (1986).
4. Rasmusson, E. M. & Carpenter, T. H. *Mon. Weath. Rev.* **110**, 354-384 (1982).
5. Philander, S. G. H. *Nature* **302**, 295-301 (1983).
6. Tsuchiya, M. *Johns Hopkins Oceanogr. Stud.* No. 4 (1968).
7. Harries, J. R. & Calf, G. E. *J. mar. Res.* **40**, 903-913 (1982).
8. Church, J. A. & Boland, F. M. *J. phys. Oceanogr.* **13**, 1746-1749 (1983).
9. Church, J. A. *Aus. J. mar. freshw. Res.* (in the press).
10. Wyrki, K. *Science* **181**, 262-264 (1973).
11. Knox, R. A. *Deep Sea Res.* **23**, 211-221 (1976).

Conclusions

The 1985-86 expeditions described here have revealed a number of major features of the western equatorial Pacific Ocean circulation. The discovery and description of the New Guinea Coastal Undercurrent, along with measurement of strong equatorward flow through Vitiaz Strait, confirms the Tsuchiya⁶ hypothesis of the origin of the high-salinity Equatorial Undercurrent waters. Time series of upper-ocean currents revealed the rapid response of the currents in the upper 100 m to the monsoonal wind forcing. A seasonally occurring, eastward-flowing surface current called the Pacific Equatorial Monsoon Jet is set up during the north-west monsoon; it is separate from the Equatorial Undercurrent. The importance of salinity in the description and dynamics of a shallower surface mixed layer is evident in the WEPOCS data and may significantly alter our view of the upper-ocean heat budget in this region.

The Western Equatorial Pacific Ocean Circulation Study is supported financially by NSF and CSIRO. This support is gratefully acknowledged, along with the assistance of Drs Richard Lambert and Angus McEwan. The captains and crews of the research vessels *Franklin*, *Moana Wave*, and *Thomas G. Thompson*, by their excellent seamanship and interest in the scientific program, were essential to the success of the field work. The technical support provided by the CSIRO Division of Oceanography, Scripps Institution of Oceanography, Woods Hole Oceanographic Institution and Oregon State University was responsible for the high quality of the WEPOCS dataset.

12. Cane, M. *Deep Sea Res.* **27**, 525-544 (1980).
13. Hansen, D. V. & Paul, C. A. *J. geophys. Res.* **89**, 10431-10440 (1984).
14. Schott, G., *Ann. Hydrogr. Mar. Met.*, **67**, 247-257 (1939).
15. Ministry of the Defence of the USSR *World Ocean Atlas: Pacific Ocean* (Pergamon, Oxford, 1974).
16. Moore, D. W. & Philander, S. G. H. *The Sea: Ideas and Observations on Progress in the Study of the Seas* (ed. Goldberg, E. D.) 319-361 (1977).
17. Garwood, R. W. Jr, Muller, P. & Gallacher, P. C. *J. phys. Oceanogr.* **15**, 1332-1338 (1985).
18. Lemasson, L. & Piton, B. *Cah. ORSTOM, Ser. Oceanogr.* **6**, 39-45 (1968).
19. Halpern, D. *Deep Sea Res.* **27**, 931-940 (1980).
20. Lukas, R. & Lindstrom, E. J. *Proc. 'Aha Huliko' a Hawaiian Winter Workshop on the Mixed Layer* (Hawaii Institute of Geophysics, University of Hawaii, in the press).

Growth hormone receptor and serum binding protein: purification, cloning and expression

David W. Leung, Steven A. Spencer, George Cachianes, R. Glenn Hammonds, Carol Collins, William J. Henzel, Ross Barnard*, Michael J. Waters* & William I. Wood

Department of Developmental Biology, Genentech, Inc., 460 Pt. San Bruno Boulevard, South San Francisco, California, USA

* Department of Physiology and Pharmacology, University of Queensland, St Lucia, Queensland 4067, Australia

A putative growth hormone receptor from rabbit liver and the growth hormone binding protein from rabbit serum have the same amino-terminal amino-acid sequence, indicating that the binding protein corresponds to the extracellular hormone-binding domain of the liver receptor. The complete amino-acid sequences derived from complementary DNA clones encoding the putative human and rabbit growth hormone receptors are not similar to other known proteins, demonstrating a new class of transmembrane receptors.

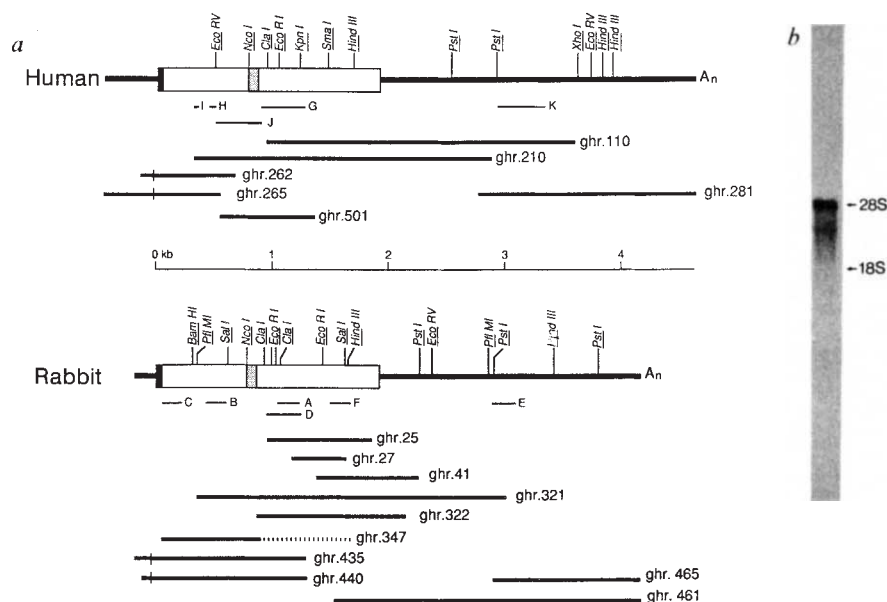
THE importance of growth hormone (GH) produced by the pituitary gland in supporting normal growth in animals has long been known, and its *in vivo* activity has been amply demonstrated by the ability of the injected hormone to restore normal growth rates in hypophysectomized animals and in GH-deficient children. Although the effects of GH have been shown *in vivo*, specifically on bone elongation, its direct action on cartilage *in vitro* has only recently been demonstrated^{1,2}. The difficulty in

demonstrating the direct effects of GH led in part to the somatomedin hypothesis in which GH stimulates the systemic release from the liver of insulin-like growth factor-I (IGF-I) which in turn stimulates cartilage and bone growth³. However, high affinity GH binding sites are found in a variety of tissues and are not localized only to the liver^{4,5}, suggesting that GH could have direct effects in addition to those produced by systemic IGF-I. The relative importance of the direct versus

GHR-N, GH receptor amino-terminal sequence. SBP-N1-3, three simultaneous amino-terminal sequences found for the serum binding protein. GHR-T4, sequence of the tryptic fragment of the GH receptor used for cDNA cloning. Sequences were determined by gas-phase sequencer analysis of electro-eluted intact proteins or of HPLC-purified tryptic fragment (S.A.S. *et al.*, manuscript in preparation). Brackets indicate residues determined from the cDNA sequence (Fig. 3). X shows residues which could not be identified. Parentheses show uncertain residues. The absence of Asn at position 28 in the serum binding protein sequence may indicate N-linked carbohydrate attached at this position.

Fig. 2 *a*, Maps of the cloned cDNAs for the human and rabbit GH receptors. The coding region of the mRNA is shown as a box. The filled region is the signal sequence; the stippled part is the transmembrane domain. A-K, Probes and oligonucleotide primers used in the cDNA cloning described below. The individual cDNA clones are labelled ghr.25-501. The clones diverge 5' of the vertical bar shown on clones ghr.262, 265, 435 and 440. The 3' divergence of clone ghr.347 is shown by a dotted line. *b*, Blot hybridization of the rabbit GH receptor mRNA. 5 µg of rabbit liver poly(A)⁺ RNA was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose⁴⁶, and hybridized to a ³²P-labelled probe prepared from the full coding region of the rabbit receptor.

Methods. Hybridizations were performed in 50% formamide overnight at 42 °C and washed in 0.2×SSC at 52–55 °C except as noted below. Fragment probes were ³²P-labelled by primed synthesis⁴⁶; oligonucleotide probes were ³²P end-labelled⁴⁶. Poly(A)⁺ rabbit liver RNA was prepared from a male New Zealand white rabbit by the LiCl precipitation method⁴⁹ followed by oligo(dT) cellulose chromatography⁴⁶. 282 µg of (A)⁺RNA were obtained from 6 g of liver. Oligo(dT) and random primed cDNAs were prepared from 2 µg of poly(A)⁺ rabbit RNA⁴⁸. These cDNAs were cloned with a λgt10 vector and hemikinased linkers as described⁴⁹. From 20 ng of oligo(dT) and 10 ng of random-primed, linked cDNA, 2×10⁶ and 1×10⁶ primary clones were obtained and amplified. These two libraries were screened with the probe ghr.3.5'GTGGAGCCATCTTCAACCAGGAGCATCTACATACCACAGAGTCCCTGACCACC, a 57-base synthetic oligonucleotide based⁵⁰ on the 19 amino-acid tryptic fragment, T4, of the receptor (Table 1). Hybridizations were performed as above with 20% formamide and washed in 1×SSC at 42 °C. Clones ghr.25, ghr.27, and ghr.41 were isolated from the random-primed library. The oligo(dT) (760,000 clones) and random primed (800,000 clones) rabbit liver cDNA libraries were rescreened with a 5'-restriction fragment from ghr.25 (probe A). Eight positive oligo(dT) and 100–300 random primed positive clones were identified. Clone ghr.321 from the oligo(dT) library was sequenced completely. The same plating of the oligo(dT) primed library was rescreened with a 5'-restriction fragment from ghr.321 (probe B). Seven positive clones were identified of which ghr.347 is shown. To facilitate isolation of long clones including the 5'-end of the gene and to take advantage of the high frequency of positive clones in the random primed rabbit library, the longer inserts from this library were isolated and recloned. The library was grown as a pool of phage, the cDNA inserts cut out with *Xho*I (a site in the cloning linker), and the long inserts (greater than 1.5 kb) isolated from an acrylamide gel. These fragments were recloned in λgt10 with *Xho*I to *Eco*RI linkers. 2×10⁶ primary clones were obtained from this library. (The complexity of this library is difficult to assess due to the pooled growth of the library to obtain DNA.) 1.1×10⁶ clones from this library were screened both with probe B (above) and with a 5' restriction fragment from ghr.347 (probe C). Many clones positive with both probes were identified of which ghr.435 and ghr.440 are shown. To obtain clones of the rabbit GH receptor 3'-untranslated region, 200,000 oligo(dT) primed rabbit liver cDNA clones were screened with a restriction fragment from ghr.321 (probe E). The hybridization was performed in 50% formamide and the filters washed in 0.2×SSC at 60 °C. Fifteen positive clones were obtained. DNA from seven of these was denatured, spotted on nitrocellulose and hybridized to an oligo (dT)₄₀ probe to isolate clones with a terminal poly(A) sequence. The hybridization was in 20% formamide and the blots were washed in 3.0 M tetramethylammonium chloride at 65 °C (ref. 51). Three of the seven clones were positive and two (ghr.461 and ghr.465) are shown. The initial human GH receptor clones were isolated from an adult liver cDNA library constructed by Lisa Coussens in the laboratory of Axel Ullrich at Genentech. This library contained oligo(dT) primed clones in a λgt10 vector. 1×10⁶ cDNA clones were screened with a restriction fragment from the rabbit clone (ghr.27, probe D). The hybridization was performed in 20% formamide and the filters washed in 1×SSC at 42 °C. Twenty eight positive clones were identified of which one, ghr.110, is shown. The same plating of the oligo(dT) primed library was rescreened with a 5-primed restriction fragment from ghr.110 (probe G). Twelve additional positive clones were identified of which one, λghr.210, is shown. To isolate clones containing the 5'-end of the hGH receptor, two additional cDNA libraries were constructed from adult human liver mRNA (provided by Karen Fisher of Genentech) as described above. One library was randomly primed, the other specifically primed with the 15-mer ghr.5 (5'-ATTGCGTGCTGCTTC, primer H). Primary clones (1.1×10⁶) from the random library and 360,000 primary clones from the specifically primed library were screened with two probes. One probe, the 27-mer ghr.65'-CATGTAGTGTAGCTTGATACAATAAG (probe I) was hybridized in 20% formamide and washed in 3.0 M tetramethylammonium chloride⁵¹. The other probe (probe C, above) was hybridized in 20% formamide and washed in 1×SSC at 42 °C. Two positives were found from the random library and nine from the specifically primed library. Clones ghr.262 (from the random library) and ghr.265 (from the specifically primed library) are shown. To obtain a second clone for the human GH receptor cDNA region between clone ghr.110 and ghr.262, 1×10⁶ clones from the random-primed human cDNA library were screened with a restriction fragment from ghr.210 (probe J). The hybridization was in 50% formamide and the filters washed in 0.2×SSC at 60 °C. Four positive clones were obtained and one (ghr.501) is shown. To obtain clones of the human 3' untranslated region, 200,000 oligo(dT) primed library clones were screened with a restriction fragment from ghr.110 (probe K). The hybridization was performed in 50% formamide and the filters washed in 0.2×SSC at 60 °C. Eleven positive clones were obtained. Three of these were tested with an oligo(dT) probe (as described above for the rabbit 3'-end clones). One clone (ghr.281) contained a poly (A)⁺ sequence.



(Fig. 1b). MAb5 has been shown to precipitate the receptor binding activity from solubilized membranes¹⁹. MAb7 and MAb263 which bind near the hormone binding site^{19,20} also specifically labelled the 130K band but the signal produced was much weaker (data not shown).

GH binding protein purification

The GH serum binding protein was purified 400,000-fold (final specific binding ~20,000 pmoles mg⁻¹) with a 14% yield by a two step procedure starting from frozen rabbit serum (Fig. 1c). The procedure used an hGH affinity column followed by a gel-filtration column to remove a contaminant that co-migrated with the serum binding protein on a non-reduced SDS gel. The final material was judged to be >70% pure based on SDS-gel analysis and a maximum theoretical binding of 20,000 pmoles mg⁻¹ assuming one GH binding-site per 51K protein.

The broad 51K major band found upon SDS-gel analysis of the purified material was identified as the GH serum binding protein in two ways. First, SDS-gel electrophoresis of the

purified material after cross-linking to ¹²⁵I-hGH shows a single diffuse radioactive band at ~75K (Fig. 1d) which corresponds to the stained band at 51K after subtracting 22K for hGH. Second, a substantial amount of the binding activity was recovered after electro-elution of the 51K band from a non-reduced SDS gel. All the recovered binding activity was localized in this region of the gel, and therefore, the purified material appears to contain one major component which specifically binds to hGH. *K_a* for hGH binding to the purified serum binding protein is 6×10⁹ M⁻¹.

Protein sequencing

The amino-terminal sequence of the liver GH receptor was determined three times, once on the MgCl₂ eluate from the GH affinity column and twice on the 130K band eluted from an SDS gel. In all three cases, two simultaneous sequences were obtained, one corresponding to the GH receptor (Table I) and the other to ubiquitin²¹. The amount of ubiquitin was 20–50% of that found for the receptor. Ubiquitin is probably covalently linked to the GH receptor by an isopeptide bond between an

Fig. 3 DNA and translated amino-acid sequence of the human and rabbit growth hormone receptor cDNA clones. Line 1, human DNA sequence. Line 2, Differences for the rabbit cDNA sequence. Line 3, the human translated amino-acid sequence. Line 4, differences for the rabbit amino acid sequence. Dashes show deletions. The DNA sequences are numbered starting with 1 at the initiating ATG, the protein sequences with the 1 at the mature amino-terminal phenylalanine. The locations of the sequenced rabbit peptides (Table 1 and S.A.S. *et al.*, manuscript in preparation) are underlined. The putative transmembrane domain is boxed. **Methods.** DNA sequence analysis was performed by the dideoxy-chain termination method using fragments cloned in the pUC118/119 vectors⁵². The human clones differ at base 558 (A in ghr.210, G in ghr.262 and 501), at base 1,124 (G in ghr.210 and 110, T in ghr.501), and at base 2,436 (C in ghr.210 and T in ghr. 110). The rabbit clones differ at base 31 (G in ghr.435 and A in ghr.440), at base 2,066 (G in ghr.321 and A in ghr.322), at base 2,102 (T in ghr.321 and 322 and C in ghr.41), and at 3,878 (G in ghr.465 and G-G in ghr.461).

Additional protein sequence data were obtained for the serum binding protein in an effort to localize the carboxy-terminus. When no further amino-terminal sequence could be obtained,

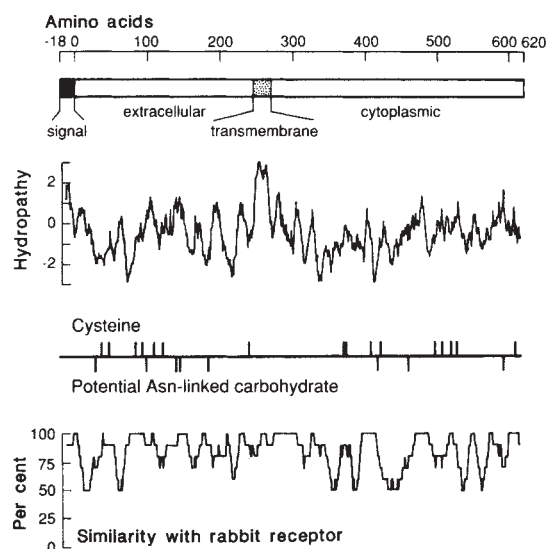


Fig. 4 Schematic of the human GH receptor. The hydropathy plot is from the method of Kyte and Doolittle⁵³ with a window of 10 residues; positive values indicate increasing hydrophobicity. The potential *N*-linked glycosylation sites are NXS/T⁵⁵. The similarity with the rabbit receptor is for exact matches over a window of 10 amino acids.

the residual protein on the sequencing filter was cleaved with cyanogen bromide and the resulting mixture sequenced. Comparison with the translated sequence of the cloned liver receptor (see below) confirmed the presence of amino acids consistent with the sequence found after the first two methionine residues, 117 (residues Val-Asp-Gln-X-X-Phe-Ser-Val-Glu-Glu-Ile-X-Gln-Pro-Asp-Pro were found) and 189 (residues Asp-Pro-X-Leu-Ser-Thr-Ser-Val were found). Some residues corresponding to the sequence following methionine 236 were present (residues Ser-Pro-Phe were found) but subsequent residues were too weak to identify this peptide with certainty. None of the sequences after the transmembrane domain were present (that is, starting at methionine 277). This mixture sequence provides additional proof for the colinearity and identity between the extracellular portion of the GH receptor and the serum binding protein. The observation that the receptor is easily proteolysed to a form of 50–60K that binds GH with high affinity and that the amino terminus of the serum binding protein has been multiply cleaved by a trypsin-like protease suggest that the GH binding protein is derived from the receptor by proteolytic cleavage.

Amino-acid sequence data were also obtained from several internal peptides of the liver GH receptor (Fig. 3). The sequence of tryptic peptide T4 (Table 1) was used to successfully clone the rabbit GH receptor cDNA.

Growth hormone receptor clones

Three approaches were used to isolate clones of the GH receptor. Before obtaining highly purified protein, MAbs¹⁹ to the rabbit liver GH receptor were used to screen both L-cells transfected with rabbit genomic DNA^{27,28} as well as rabbit liver cDNA clones in a λ gt11 vector²⁹. No GH receptor clones were isolated by either of these two approaches.

Rabbit growth hormone receptor clones were isolated by screening liver cDNA libraries with a single 57-mer oligonucleotide probe based on the 19-residue amino-acid sequence of a tryptic fragment of the receptor (Table 1). From a screen of 100,000 oligo(dT) and 100,000 random-primed clones, 2 and 27 positive clones respectively, were identified. Some of these clones were mapped and sequenced (Fig. 2a, ghr.25, ghr.27 and ghr.41). The translated DNA sequence matched exactly the 19 amino-acid sequence of the tryptic fragment used for the probe,

and the nucleotide sequence of the probe matched the cloned DNA sequence in two 14-base regions separated by a single mismatch. This tryptic fragment starts at amino acid 538 near the carboxy terminus of the protein (Fig. 3). Clones of the entire coding and 3'-untranslated regions were isolated by rescreening the same libraries with fragment probes obtained from the initial set of clones (see legend to Fig. 2).

Human growth hormone receptor clones were isolated by screening a human liver cDNA library with a restriction fragment probe from one of the first rabbit clones isolated (ghr.27). Overlapping clones of the entire human coding and 3' untranslated regions were isolated from oligo(dT), random, and specifically primed liver cDNA libraries. Figure 2a shows the map of a selected number of both the rabbit and human GH-receptor cDNA clones. The complete cDNA and translated protein sequences of both the rabbit and human clones are shown in Fig. 3.

Two independent clones were isolated for all the coding and for much of the untranslated regions of both the rabbit and human cDNAs. The DNA sequences of these pairs of clones match, with the exception of a few single base differences (see legend to Fig. 3) and some considerable divergence in the 5'-untranslated region (described below). Only two of the differences affect the amino-acid sequence. In the human GH receptor, a G to T difference in ghr.501 changes serine 357 to isoleucine, and in the rabbit receptor an A to G difference in ghr.440 changes alanine minus 8 to threonine.

In the 5'-untranslated region beginning 12-base pairs (bp) 5' of the initiating ATG codon, most of the clones isolated diverge completely. Of the six human and three rabbit cDNA clones that include this 5' region, only two pairs are similar. The two human clones shown in Fig. 2a (ghr.262 and ghr.265) are identical in this region, and one rabbit clone (ghr.435) and one human clone (ghr.244, not shown) are 79% identical. The remaining five clones are unique. Probably these clones are the result of multiple RNAs which are differentially spliced at the 5' end. The observation that clones from two different species isolated from a number of different cDNA libraries diverge at the same point suggests that this is not a cloning artifact. The biological significance of these differentially spliced clones is being investigated. Clones containing alternatively spliced 5'-untranslated regions have been isolated for a number of other eukaryotic genes^{30,31}.

Two independent rabbit clones diverge from the others 3' of bp 877 (one, ghr.347, is shown in Fig. 2a). The divergent sequence of these two clones is identical. The translated amino-acid sequence of these two clones diverges from the others beginning four residues carboxy-terminal of the putative transmembrane domain (see below) and includes four new amino acids before encountering a stop codon. Thus, these two clones would encode a protein containing the extracellular GH binding domain, the transmembrane domain, and a truncated cytoplasmic domain of only eight residues. If this protein is synthesized, it would probably show altered intracellular signalling properties. Truncated growth factor receptors have been shown to function as oncogenes conferring the transformed phenotype on cultured cells³². Alternatively, this truncated protein could be a specific precursor for the secreted serum binding protein.

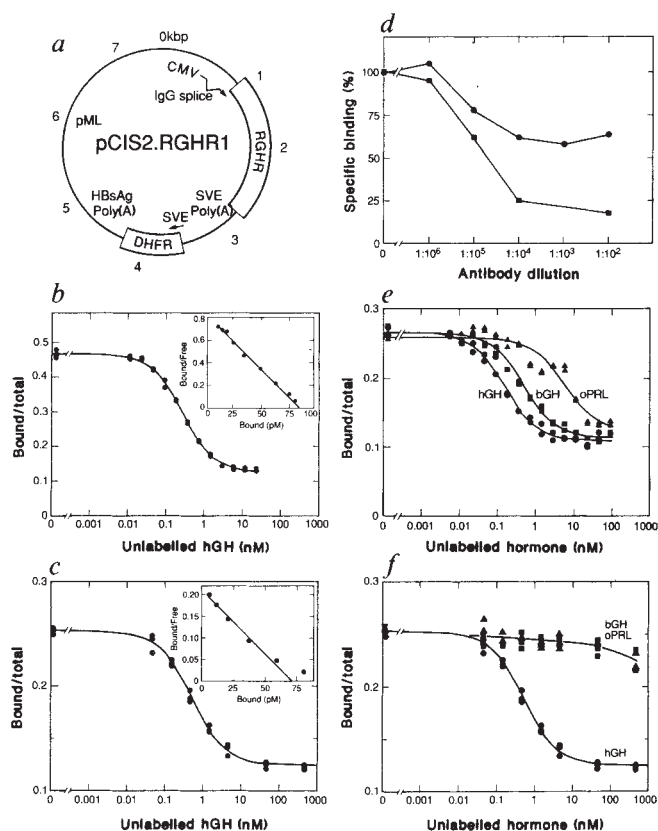
Two of the rabbit cDNA clones (ghr.461 and 465) and one human cDNA clone (ghr.281) contain poly(A) tracts at the 3' ends. These are preceded by AATAAA (human) and CATAAA (rabbit) poly(A) addition signals. Blot hybridization of poly(A⁺) rabbit liver RNA with a full-length rabbit probe shows one main hybridizing band of ~4,700 bp, approximately the size of the cloned cDNA (Fig. 2b).

Amino-acid sequence

Both the rabbit and human growth hormone receptor clones contain an open reading frame of 638 amino acids. All 11 amino-terminal, tryptic, and V8 peptide sequences determined

Fig. 5 Expression of the rabbit and human GH receptor cDNA clones in COS-7 monkey kidney cells^{54,55}. *a*, Mammalian expression vector, pCIS2.RGHR1, for full-length rabbit GH receptor. *b-f*, Assays of expressed GH receptor and binding protein by competition with ¹²⁵I-hGH. No specific binding was observed in untransfected COS-7 cells. *b*, Competition assay with unlabelled hGH of membranes from pCIS2.RGHR1 transfection expressing rabbit GH receptor. Insert, Scatchard plot of the same data. *c*, Competition assay with unlabelled hGH of culture medium from pCIS2.sHGHR transfection expressing human soluble binding protein. Insert, Scatchard plot of the same data. *d*, Competition assay with unlabelled MAb 263 (●) or 7 (■) of rabbit receptor in B (performed as described²⁰ but with hGH as the excess unlabelled hormone). *e*, Competition assay with unlabelled hGH (●), bovine GH (■), or ovine prolactin (▲) of rabbit receptor in *b*. *f*, Competition assay with unlabelled hGH (●), bovine GH (■), or ovine prolactin (▲) of human binding protein in *c*.

Methods. The full-length rabbit GH receptor cDNA sequence was assembled into a mammalian expression vector previously used for the expression of factor VIII (pF8CIS9080)⁵⁶. In this vector transcription of the receptor is directed by a human cytomegalovirus promoter (CMV). The plasmid was constructed by joining the *Cla*I site in the vector to the *Ava*II site 12-bp 5' of the initiation ATG in ghr.435 with a short synthetic oligonucleotide. Clone ghr.435 was joined to ghr.321 at the *p*III site (Fig. 2) to assemble the full coding region. The 3'-end was constructed by joining the *Dra*I site 207-bp 3' of the stop codon to the *Hpa*I site in the vector, to generate pCIS2.RGHR.1. A similar vector, pCIS2.sHGHR, was constructed for the soluble, secreted form of the hGH receptor. At the 5'-end the *Cla*I site in the vector was joined to the *Ava*II site 12-bp 5' of the initiating ATG in ghr.262 as described above. Clone ghr.262 was joined to ghr.210 at the *Eco*RV site to assemble the extracellular domain. The receptor was artificially truncated at amino acid 247 by use of an approximately 70 bp synthetic oligonucleotide to reconstruct the carboxy-terminal portion of the extracellular domain beginning at the *Hgi*AI site at amino acid 228 and to introduce a stop codon followed by a *Hpa*I restriction enzyme site. This *Hpa*I site was joined to the *Hpa*I site in the vector. Competition assays were performed by incubation for 24 h at room temperature of the unlabelled competing ligand, ¹²⁵I-hGH (~30,000 c.p.m., 50–100 µCi µg⁻¹), and the diluted sample in 0.5 ml final volume of assay buffer (50 mM Tris HCl, pH 7.4 containing 10 mM CaCl₂, 0.1% bovine albumin and 0.02% NaN₃). Assays were terminated by addition of 0.5 ml 0.1% bovine γ-globulin followed by 1 ml 30% polyethylene glycol 8,000 (both at 4 °C in phosphate buffered saline containing 0.02% NaN₃). Bound ¹²⁵I-hGH was determined by centrifugation at 4,000 g for 20 min and counting the pellets. For the soluble human binding protein, MAb263 was included in the assay mixture at 1/2000 final dilution to make the labelled complex PEG-precipitable.



from the purified rabbit receptor are present in this translated region (see Fig. 3). The amino-terminal amino-acid sequence determined from the purified rabbit receptor is preceded by 18 amino acids beginning with a methionine. These 18 residues contain a hydrophobic core of 14 amino acids flanked by charged residues indicative of a membrane signal sequence³³. The DNA sequence surrounding the ATG codon encoding the methionine matches the consensus sequence expected for a translation initiation site³⁴. Thus the mature form of the receptor would be expected to have 620 amino-acid residues and have a *M_r* of 70K. This is smaller than the 130K determined by SDS-gel analysis for the purified rabbit liver GH receptor, but much of the apparent discrepancy can be accounted for by glycosylation and covalently-bound ubiquitin (~9K). (Treatment of the purified rabbit receptor with glycosidases reduces the molecular weight determined by SDS-gel analysis from 130K to ~95K, S.A.S., unpublished data).

A hydropathy plot of the hGH receptor sequence (Fig. 4) shows only one major hydrophobic region of ~24 residues centrally located in the molecule. This is presumably the transmembrane domain which separates the extracellular, amino-terminal, GH binding domain from the intracellular, carboxy-terminal, signalling domain. The potential *N*-linked glycosylation sites (NXS/T)³⁵ (five in the extracellular domain) as well as the locations of the cysteine residues are shown in Fig. 4. There are no regions with a high serine or threonine content, often indicative of *O*-linked glycosylation³⁶.

The rabbit and human GH-receptor protein sequences are highly similar throughout with 84% amino acid identity (Figs 2 and 4). The two sequences can be aligned without any inser-

tions or deletions. The similarity extends throughout the molecules including the signal sequence and the transmembrane domain. Computer assisted searches of several data bases have found no clearly significant similarity to any other protein (Protein Identification Resource, National Biomedical Research Foundation, Georgetown Univ. Med. Center, Washington, DC, 20007, USA and GenBank, Bolt, Beranek and Newman, Inc., Cambridge, Massachusetts 02238 USA). The receptor is not related to the known tyrosine-kinase growth factor receptors³⁷ and contains no internally repeated domains or particularly cysteine-rich regions in the extracellular domain (Fig. 4), as has been found with some other receptors^{38,39}. The seven extracellular cysteines are at the same position in the human and rabbit sequences. Of the nine intracellular cysteines in the human receptor and eight in the rabbit receptor, seven are at the same positions.

Amino-terminal sequence data (Table 1) show that the soluble growth hormone binding protein found in rabbit serum has the same amino-acid sequence as the membrane-bound receptor. The cyanogen bromide digest results (above) suggest that the binding protein sequence extends to at least methionine 236. This protein could be released by proteolysis of the membrane bound receptor near the transmembrane domain, or alternatively it could be the product of the truncated clone described earlier.

Expression

A full-length cDNA for the rabbit GH receptor was assembled in a mammalian expression vector (Fig. 5a) containing a cytomegalovirus promoter and the other functions necessary for high level expression in mammalian cells. Transient transfection

of the plasmid pCIS2.RGHR1 in COS-7 monkey kidney cells results in the expression of membrane-bound receptor. Binding experiments (Fig. 5b) with ^{125}I -labelled hGH show that the expressed receptor has a high affinity ($K_a = 10 \times 10^9 \text{ M}^{-1}$), comparable to that of the purified rabbit receptor ($K_a = 10\text{--}30 \times 10^9 \text{ M}^{-1}$). About 200,000 copies of the receptor are expressed per cell. Small amounts of high-affinity GH binding activity are also found in the culture medium from these cells (data not shown). This soluble binding activity is probably the same or similar to the released extracellular domain of the receptor found in rabbit serum.

Figure 5e shows the displacement of ^{125}I -hGH from the expressed rabbit receptor by bovine growth hormone and ovine prolactin. As expected for the rabbit somatogenic receptor, all three hormones can displace ^{125}I -hGH completely. But for 50% displacement about 3–5-fold higher concentrations of bovine GH and about 100-fold higher concentrations of ovine prolactin are required as compared with hGH.

Previous experiments with a panel of MAbs to the rabbit GH receptor had identified three types of receptor based on full and partial antibody competition for GH binding²⁰. Competition between ^{125}I -hGH and two of these MAbs for binding to the rabbit receptor expressed in COS cells shows that at saturating levels MAb263 competes for about 40% of hGH binding and MAb7 competes for about 80% (Fig. 5d). These and other data (M.J.W., unpublished data) suggest that the expressed rabbit receptor includes at least two of the GH receptor types defined previously (types 1 and 2), showing that a single gene can encode multiple receptor types defined by differential antibody binding. These types could be due to post-translational modification of the receptor or some other form of receptor processing. One of these antibodies, MAb263, is of special interest because it recognizes a receptor epitope which is similar in several species^{14,20} and because it is a GH agonist and mimics the biological activity of GH in promoting fibroblast DNA synthesis⁴⁰. The binding of this agonist MAb to the expressed receptor provides more evidence that this is the protein required to transduce the GH signal in target cells.

Expression in tissue culture of the full-length human GH receptor has been hampered by difficulties in growing the assembled full-length cDNA clones in *Escherichia coli*, but expression of the extracellular GH binding domain of the human receptor has been accomplished. The human receptor has been

genetically engineered to express only the extracellular GH binding domain by substitution of a stop codon at the first amino acid of the transmembrane domain (amino acid 247). As this protein still contains the natural signal sequence, we expected it to be secreted from transfected cells and released into the culture medium, resulting in a soluble GH binding protein similar to the GH binding protein found in human serum^{16,17}. Transient transfection in COS-7 cells of a mammalian expression vector similar to that used for the full-length rabbit receptor (Fig. 5a), but containing the truncated hGH binding domain, results in secretion into the culture medium of a GH binding activity. Binding experiments (Fig. 5c) show that this soluble protein has a high affinity for hGH ($K_a = 3 \times 10^9 \text{ M}^{-1}$) and is expressed at 55 ng ml^{-1} in the culture medium. As expected for the hGH receptor, bovine GH and ovine prolactin do not compete for ^{125}I -hGH binding to this protein (Fig. 5f).

Conclusions

The purification and protein sequence data presented here establish the structural identity of the GH serum binding protein as the extracellular, hormone-binding domain of the membrane-bound GH receptor. This work has formed the basis for the cloning and expression of the receptor cDNA. Final proof that these cloned receptors transduce the signal for growth in mammals will require the demonstration of an appropriate biological effect in transfected cells, as well as some genetic analysis of receptor deficient animals or human populations. Few suitable cell-based biological assays yet exist for GH and no intracellular signal has been identified^{4,5}. Human populations that are potential candidates for defective or modified GH receptors include Laron dwarfs⁴¹ and African pygmies⁴². Both these populations have normal GH levels, but reduced IGF-I levels, at least under some conditions. Human growth hormone binding activity is absent in both the liver⁴³ and serum^{44,45} of Laron dwarfs, providing important evidence that the receptor cloned here is central to the growth process. It will also be important to determine whether the GH receptor gene is unique. High affinity GH binding has been found on a number of different cell types, and the possibility remains that this receptor is only one of several required to transmit the signal for proper growth.

We thank Hugh Niall for his support and Jim McCabe for help in this work. Portions of this work were supported by an NHMRC grant [322] to M.V.W. and by Genentech.

Received 18 September; accepted 22 October 1987.

- Nilsson, A. *et al. Science* **233**, 571–574 (1986).
- Lindahl, A., Isgaard, J., Nilsson, A. & Isaksson, O. G. P. *Endocrinology* **118**, 1843–1848 (1986).
- Salmon, W. D. Jr & Daughaday, W. H. *J. Lab. clin. Invest.* **49**, 825–836 (1957).
- Hughes, J. P. & Friesen, H. G. A. *Rev. Physiol.* **47**, 469–482 (1985).
- Isaksson, O. G. P., Edén, S. & Jansson, J.-O. A. *Rev. Physiol.* **47**, 483–499 (1985).
- Zezulak, K. M. & Green, H. *Science* **233**, 551–553 (1986).
- Rechler, M. M., Nissley, S. P. & Roth, J. N. *Engl. J. Med.* **316**, 941–942 (1987).
- Waters, M. J. & Friesen, H. G. *J. biol. Chem.* **254**, 6815–6825 (1979).
- Tsushima, T. *et al. FEBS Lett.* **147**, 49–53 (1982).
- Haepfle, M.-T., Aubert, M. L., Djiane, J. & Kraehenbuhl, J.-P. *J. biol. Chem.* **258**, 305–314 (1983).
- Donner, D. B. *J. biol. Chem.* **258**, 2736–2743 (1983).
- Carter-Su, C., Schwartz, J. & Kikuchi, G. *J. biol. Chem.* **259**, 1099–1104 (1984).
- Hughes, J. P., Simpson, J. S. A. & Friesen, H. G. *Endocrinology* **112**, 1980–1985 (1983).
- Asakawa, I. K. *et al. Biochem. J.* **238**, 379–386 (1986).
- Ymer, S. I. & Herington, A. C. *Molec. cell. Endocrinol.* **41**, 153–161 (1985).
- Baumann, G., Stolar, M. W., Amburn, K., Barsano, C. P. & DeVries, B. C. *J. clin. Endocr. Metab.* **62**, 134–141 (1986).
- Herington, A. C., Ymer, S. I. & Stevenson, J. L. *J. clin. Invest.* **77**, 1817–1823 (1986).
- Barnard, R. & Waters, M. J. *Biochem. J.* **237**, 885–892 (1986).
- Barnard, R., Bundesen, P. G., Rylatt, D. B. & Waters, M. J. *Endocrinology* **115**, 1805–1813 (1984).
- Barnard, R., Bundesen, P. G., Rylatt, D. B. & Waters, M. J. *Biochem. J.* **231**, 459–468 (1985).
- Schlesinger, D. H., Goldstein, G. & Niall, H. D. *Biochemistry* **14**, 2214–2218 (1975).
- Goldknopf, I. L. & Busch, H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 864–868 (1977).
- Siegelman, M. *et al. Science* **231**, 823–829 (1986).
- Yarden, Y. *et al. Nature* **323**, 226–232 (1986).
- Baxter, R. C. *Endocrinology* **117**, 650–655 (1985).
- Gorin, E. & Goodman, H. M. *Endocrinology* **116**, 1796–1805 (1985).
- Littman, D. R., Thomas, Y., Maddon, P. J., Chess, L. & Axel, R. *Cell* **40**, 237–246 (1985).
- Kuhn, L. C., Barbosa, J. A., Kamarc, M. E. & Ruddle, F. H. *Molec. Biol. Med.* **1**, 335–352 (1983).
- Huynh, T. V., Young, R. A. & Davis, R. W. *DNA Cloning* vol. I (ed. Glover, D.) 49–78 (IRL, Oxford, 1985).
- Peralta, E. G. *et al. Science* **236**, 600–605 (1987).
- Adelman, J. P., Mason, A. J., Hayflick, J. S. & Seeburg, P. H. *Proc. natn. Acad. Sci. U.S.A.* **83**, 179–183 (1986).
- Coussens, L. *et al. Nature* **320**, 277–280 (1986).
- Perlman, D. & Halvorson, H. O. *J. molec. Biol.* **167**, 309 (1983).
- Kozak, M. *Nucleic Acids Res.* **12**, 857–872 (1984).
- Marshall, R. D. A. *Rev. Biochem.* **41**, 673–702 (1972).
- Russell, D. W. *et al. Cell* **37**, 577–585 (1984).
- Yarden, Y. *et al. Nature* **323**, 226–232 (1986).
- Ullrich, A. *et al. Nature* **309**, 418–425 (1984).
- Yamamoto, T. *et al. Cell* **39**, 27–38 (1984).
- Cook, J. J., Haynes, K. M., Barnard, R., Waters, M. J. & Werther, G. A. *Proc. Endocrine Soc. U.S.A.* **69**, 618 (1987).
- McKusick, V. A. *Mendelian Inheritance in Man*, 6th edn, 895–896 (Johns Hopkins University Press, Baltimore, 1983).
- Merimee, T. J., Zapf, J., Hewlett, B. & Cavalli-Sforza, L. L. *N. Engl. J. Med.* **316**, 906–911 (1987).
- Eshet, R., Laron, Z., Pertzalan, A., Arnon, R. & Dintzman, M. *Israel J. med. Sci.* **20**, 8–11 (1984).
- Daughaday, W. H. & Trivedi, B. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4636–4640 (1987).
- Baumann, G., Shaw, M. A. & Winter, R. J. *clin. Res.* **35**, 582A (1987).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning. A Laboratory Manual* (Cold Spring Harbor, New York, 1982).
- Cathala, G. *et al. DNA* **2**, 329–335 (1983).
- Gubler, U. & Hoffman, B. J. *Gene* **25**, 263–269 (1983).
- Wood, W. I. *et al. Nature* **312**, 330–337 (1984).
- Lathe, R. J. *molec. Biol.* **183**, 1–12 (1985).
- Wood, W. I., Gitschier, J., Lasky, L. A. & Lawn, R. M. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1585–1588 (1985).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
- Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105–132 (1982).
- Gluzman, Y. *Cell* **23**, 175–182 (1981).
- Gorman, C. *DNA Cloning* Vol. II (ed. Glover, D.) 143–190 (IRL, Oxford, 1985).
- Eaton, D. L. *et al. Biochemistry* **25**, 8343–8347 (1986).

Hard X-ray images of the galactic centre

G. K. Skinner, A. P. Willmore, C. J. Eyles, D. Bertram, M. J. Church, P. K. S. Harper, J. R. H. Herring, J. C. M. Peden, A. M. T. Pollock*, T. J. Ponman & M. P. Watt

Department of Space Research, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

The centre of our Galaxy has been observed extensively at infrared and radio wavelengths. The compact radio source at the nucleus (Sgr A*) may be a weaker form of those in active galaxies. The region is obscured in the visible band by ~ 30 magnitudes of absorption but the intervening dust and gas clouds become transparent again for X-rays of energy greater than a few keV. We report here observations made with a coded mask X-ray telescope flown on the Spacelab 2 mission (29 July to 6 August 1985), yielding for the first time images of the galactic centre in high-energy X-rays up to 30 keV. Components detected include a region of diffuse emission 2° in diameter and several new point sources. At the higher energies emission from the nucleus is weak and the region is dominated by one of the surrounding point sources.

The only detailed X-ray image of the galactic centre region until now was obtained by Watson *et al.*¹ in 1979 with the Einstein Observatory, using X-rays with energy < 4.5 keV (ref. 1, referred to below as WWGH). Twelve point sources were seen in the $\sim 1^\circ$ field, together with diffuse emission $\sim 33 \times 18$ arc min in extent. With the exception of a rocket-borne prototype of the instrument used for the measurements reported here², the only previous observations at energies above ~ 4 keV have been with non-imaging instruments (for example, refs 3–6). These have indicated only one persistent source (A1742–294) although at least two transients^{3,7,8} have been observed (one, A1742–289, within 1 arc min⁹ of Sgr A*) and there has been evidence for a complex of other sources in the region.

The observations reported here were designed to complement WWGH and to explore the structure of the region and the origin of the emission at X-ray energies up to 30 keV. Part of the region surveyed was also observed by Kawai *et al.*¹⁰ with a slit scanning instrument on the SPARTAN-1 mission about six weeks before our measurements and their data, although not extending to such high energies, provide a nearly contemporary comparison.

The new images reveal a region of diffuse emission 5 to 10 times larger than that seen in the Einstein observations. A number of new point sources were detected. At the lowest energies, which overlap those of the Einstein observations, emission is detectable from the immediate vicinity of the galactic nucleus. Although stronger than expected from the results of WWGH, the nucleus is not an important source of emission at energies greater than 20 keV, where the region is dominated by 1E1740.7–2942, a source ~ 50 arc min away. This suggests that the strong variable flux at even higher energies (~ 100 keV), frequently reported from the general vicinity of the galactic centre, may not be associated with activity in the galactic nucleus.

The coded mask telescope (SL2-XRT) built by the University of Birmingham¹¹ is a dual instrument, each of the two units imaging the same 6° -square field of view. Different mask designs were used to give different resolutions in the otherwise identical units. The nominal resolution of the 'coarse' telescope was 12 arc min full width at half maximum (FWHM), and of the 'fine'

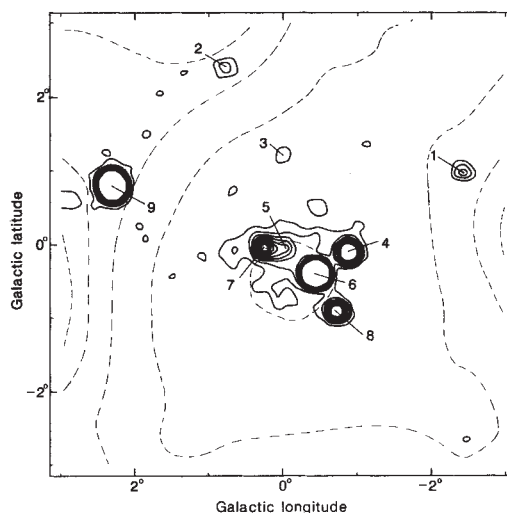


Fig. 1 Image in 3–30 keV X-rays obtained with the coarse telescope. Solid contours show intensity relative to the local noise level and are at 2σ , 4σ , 6σ , ..., 18σ . Dotted lines show the variation of noise level over the images. Absolute intensities are thus the product of the two levels. Noise-level contours increase outwards from the centre and are at 1σ noise levels of 0.06, 0.12, 0.18, 0.24, 0.30 SL2-XRT counts s^{-1} per 'sub-pixel' (sub-pixel is here used to refer to an element smaller than the resolvable pixels; sub-pixels are 6 arc min square for the coarse telescope, 1.5 arc min square for the fine). Nominal resolution is 12 arc min FWHM, but a gaussian filter of 4 arc min FWHM has been used to clean up the contours. Numbers identify sources in Table 1. Some noise peaks appear at the 2σ level, as expected.

telescope 3 arc min FWHM. The telescopes were designed to operate in the energy range 2.5–25 keV, although useful sensitivity was obtained to greater than 30 keV. The method of operation of the telescope and the data analysis techniques will be described in detail elsewhere (some of the methods are discussed in ref. 12 and references therein). The analysis results in images with statistically independent 'sub-pixels' sampled on a grid somewhat finer than the instrument resolution. A grid spacing of half the instrument FWHM was used here.

The images presented here are combinations of component images obtained in a large number of short observations spread through the eight-day Spacelab 2 mission. About seven hours of data contribute, including some obtained when the telescope was not pointed directly at the galactic centre but at adjacent regions. An image of a 6° -square region obtained with the coarse telescope using photons from 3.0 to 30 keV and centred on the origin of galactic longitude and latitude is shown in Fig. 1. The dotted contours show how the sensitivity varies over the field of view because of the different pointing directions and the effects of a 3.1° FWHM collimator which forms part of each telescope. The units are SL2-XRT counts s^{-1} ; 1 count s^{-1} is approximately 3.4×10^{-11} erg $cm^{-2} s^{-1}$, or $0.51 \mu Jy$, in the energy band 3–30 keV for a source with a typical spectrum.

The central 2° -square region of the corresponding fine telescope image is shown in Fig. 2. (The remainder of this image shows only features visible in Fig. 1.) Because the energy and time of each photon was telemetered, a spectrum or a light curve can be obtained from any point in the field of view. We have not yet analysed the available temporal or spectral data in detail, but the dramatic differences in the spectra of the various components are apparent from comparison of Fig. 3, which shows a coarse telescope image from events in the energy range 19–30 keV, with Fig. 1. In view of these differences, the conversion from observed counting rate to flux has been based on preliminary spectral fits and thus differs between sources.

Consistent with the Einstein images and with Ariel 5 and

* Present address: Space Science Department, ESTEC, Postbus 299, 2200AG, Noordwijk, Netherlands.

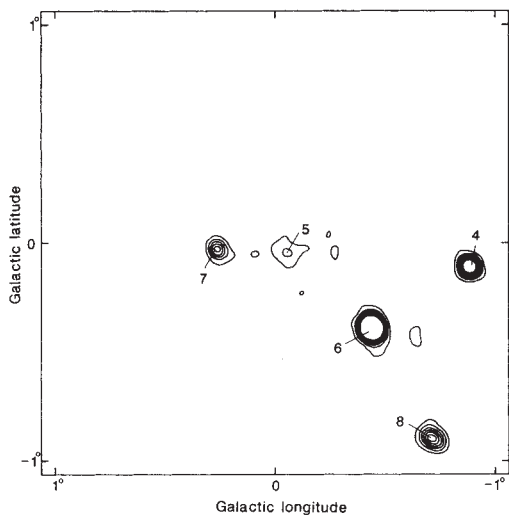


Fig. 2 Central part of fine telescope image. As Fig. 1, except for nominal resolution of 3 arc min FWHM, and filter 1 arc min FWHM.

SAS-3 non-imaging data, the group of point sources around the galactic centre is dominated by A1742-294, ~ 30 arc min from the nucleus. We find that in the 4.25-15 keV range this source accounts for 37% of the X-ray flux from the central 1.3° radius. Its intensity during the SL2 flight is similar to that observed with Ariel 5⁵, SAS-3⁶ and two rocket instruments^{2,4} and it seems likely that this source was responsible for the majority of the flux originally detected by Uhuru³, although at least two other sources must have contributed to explain the extent of the emission.

The individual source components detectable in Figs 1 and 2 are listed in Table 1. Here and elsewhere a distance of 10 kpc is assumed. There are three sources, indicated by 1E numbers, which can be identified with ones first seen in Einstein data. For these sources, and in other cases where data are available, we show (column 8) the SL2-XRT count rate expected based on the Einstein flux and the spectrum adopted by WWGH (thermal bremsstrahlung at $kT = 5$ keV, with absorption due to a hydrogen column density of 6×10^{22} atoms cm^{-2}). In each case the observed count rate is much higher than predicted.

One of the sources in common with the Einstein observations is 1E1742.5-2859, which is coincident with Sgr A*, the radio source at the galactic nucleus. It appears as an extension to 1E1743.1-2843 in Fig. 1, and as a well-resolved source in the fine telescope image (Fig. 2). The intensity is a factor of eight higher than expected on the basis of WWGH. We have examined the profile of the image to investigate whether it could be explained as the peak of a region of diffuse emission, but find it to be entirely consistent with a point source at the resolution of the fine telescope (nominally 3 arc min FWHM, broadened to 4.2 arc min by finite detector resolution and reconstruction uncertainties, as seen for known point sources).

1E1740.7-2942, identified with Source 4, lies outside the image of WWGH but was present in a separate Einstein observation analysed by Hertz and Grindlay as part of their galactic plane survey¹³. This is the only source they list in the 6° region covered in the present work, except for A1742-294 and SAO185635, a nearby G5 star not expected to emit appreciably at our higher energies. 1E1740.7-2942 is notable for a remarkably hard spectrum, and dominates the high-energy image (Fig. 3).

We identify SLX1732-304 with the globular cluster Terzan 1. Although no continuous flux has been reported from this cluster, it has been proposed by Makishima *et al.*¹⁴ as the origin of two X-ray bursts. In their survey of X-ray sources in globular clusters¹⁵ Hertz and Grindlay found a bimodal luminosity function with two distinct classes having 0.4-4.5 keV luminosities

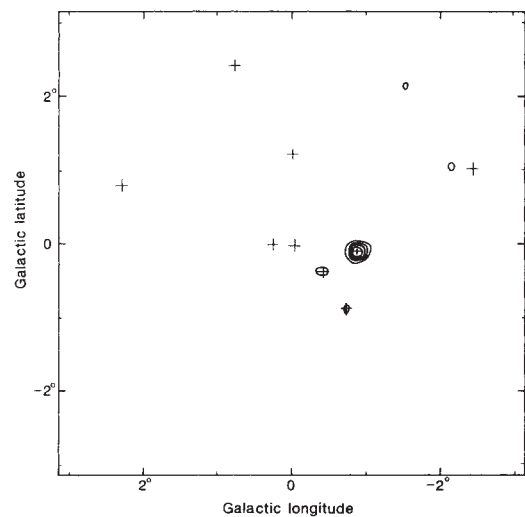


Fig. 3 As Fig. 1, except that X-ray energy range is restricted to 19-30 keV. Crosses show positions of sources in Fig. 1.

$< 10^{34.5}$ and $> 10^{36}$ erg s^{-1} . They associate these with white dwarf and neutron star binaries respectively¹⁶. We estimate an apparent luminosity of 3×10^{36} erg s^{-1} in their band, making it clearly a member of the second group, consistent with identification with a burst source.

Three other previously unreported sources are apparent in the images and listed in Table 1. Their intensity is such that if they occurred elsewhere in the Galaxy they would have been detected in surveys. Slit-scanning instruments such as Uhuru and the Ariel 5 sky survey instrument tend to be confused in complex regions, so it is possible that these are persistent sources which have previously been missed. One of them (SLX1744-299) is also apparent in the data obtained by Kawai *et al.*¹⁰ using Spartan, six weeks before our observations. The position of another (SLX1737-282) lies within one of the fields of the Einstein galactic plane survey (Seq. 2521, 22 September 1979). We have re-examined these data and find the source clearly present but very close to one of the ribs supporting the window, presumably explaining why it was not picked out by the automatic search algorithms, and not listed by Hertz and Grindlay.

As part of this re-examination of the Einstein database we have extracted 'Rev B' positions for some of the sources seen in the SL2 data. These positions, given in Table 1, are either new or superseded previously published data.

In Table 1 we list also upper limits for the transient 4U1735-28 (GX359+2)^{3,7} and for GX+0.2, -0.2. The latter was not detected by WWGH but was reported previously in three studies of the galactic centre in 1976-7^{2,4,5}, at about 80 times our upper limit, suggesting that it too was a transient. The table contains all sources detected so far, using a strict set of criteria requiring a 3σ detection in each telescope together with a satisfactory fit to the instrument point spread functions. Inside the third contour of Fig. 1 we believe the search to be complete to a level in the range $(1.5-3) \times 10^{-11}$ erg $\text{cm}^{-2} \text{s}^{-1}$.

The resolution of one of the two telescopes was made comparatively coarse to give a higher sensitivity to weak diffuse emission. Detailed examination of the data presented in Fig. 1 reveals that the diffuse emission which is seen surrounding the central sources extends to about 1° from the galactic nucleus. To improve the statistical significance of this faint area, the intensities were combined into radial bins of 12 arc min (Fig. 4). A more restricted energy range (4.25-15 keV) was used to avoid any possible instrumental effects and to improve the signal-to-noise ratio. Circular regions of radius 12-16 arc min (depending on the source intensity) centred on each of the point sources in Table 1 were excluded. The emission is bounded by a rather sharp drop at a radius of 72 arc min. The variation along

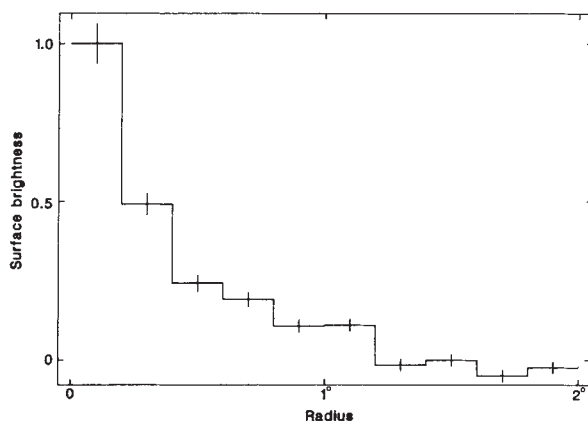


Fig. 4 Surface brightness of the diffuse X-ray component, in radial bins centred on Sgr A*. Energy range is 4.25–15 keV. Ordinate is relative to the first bin, which has a level of 9.2×10^4 SL2-XRT counts $\text{s}^{-1} \text{sr}^{-1}$.

and across the plane was investigated, confirming that the outer boundary is roughly circular. Within this is a brighter, elliptical region, approximately centred on Sgr A*, though a displacement of a few arc min cannot be excluded. The total emission from the diffuse region is 27 ± 1.3 counts s^{-1} , corresponding to an energy flux (in 4.25–15 keV) of 7.0×10^{-10} erg $\text{cm}^{-2} \text{s}^{-1}$, or to a luminosity of 8.3×10^{36} erg s^{-1} . The mean surface brightness is 5.1×10^{-7} erg $\text{cm}^{-2} \text{s}^{-1} \text{sr}^{-1}$ and the peak 2.4×10^{-6} erg $\text{cm}^{-2} \text{s}^{-1} \text{sr}^{-1}$.

Although we detect emission from the galactic nucleus (or its immediate surroundings) at a level seven times greater than would be expected based on earlier lower energy measurements, the implied luminosity is only 6.2×10^{35} erg s^{-1} . There have been numerous reports of strong, variable, high-energy X-ray (~ 15 –150 keV) emission from the galactic centre region^{17–19}. In the HEAO-1 A-4 survey¹⁷ a source within 2° of the nucleus was seen with a 80–180 keV flux implying that (assuming a distance of 10 kpc) it is the most luminous persistent source in the sky at these energies. It has naturally been suggested that the source is associated with the galactic nucleus but our observations make this seem unlikely. Such an identification would require a spectrum rising with a photon index $\alpha > 0.5$ to connect our observations at 25 keV to those at 100 keV (we use the spread of 100 keV fluxes from the five observations^{17,18,20} with instruments of

resolution $\leq 5^\circ$). Identifying the high-energy emission with A1742–294 (ref. 18) is slightly more plausible (implying $\alpha = -0.1$ to -0.6), but we suggest that the most likely origin is 1E1740.7–2942 ($\alpha = -1.0$ to -1.5). Variable 511-keV positron annihilation-line radiation^{21–24} and strong continuum flux extending to > 2 MeV^{19,23} are also known to come from directions consistent with the galactic centre. In these cases the direction is known only to $\sim 5^\circ$, but it is tempting to speculate that these may be associated with the ~ 100 keV flux and hence perhaps with 1E1740.7–2942.

The systematic difference between the observed count rates and those expected on the basis of Einstein measurements implies either that the intensities have changed or that the assumed WWGH spectrum is in error. It is unlikely that the four brightest sources in Einstein observations of the region were simultaneously still brighter at the time of the Spacelab measurements. The results from the Einstein monitor proportional counter (MPC) support this conclusion. The MPC had no spatial resolution within its 0.75° (FWHM) field of view but had an energy range more like that of the SL2 instrument (1.5–20 keV) and was collecting data during the observations analysed by WWGH. We find that the MPC count rates, too, are much higher than would have been expected on the basis of the IPC spectra.

Table 1 Positions and intensities of point sources

Source*	Position† RA h.m.s.	Dec d.m.s.	Error radius (90% confidence) (arc min)	Count rate‡ (s^{-1})	Flux§ (10^{-11} erg $\text{cm}^{-2} \text{s}^{-1}$)	Luminosity (10^{36} erg s^{-1})	Expected¶ count rate (s^{-1})
1 SLX1732–304	17 32 39	–30 25 41	1.6	5.1 ± 0.38	18.4	2.2	
2 SLX1735–269	17 35 08	–26 58 34	2.0	5.4 ± 0.53	23.4	2.8	
3 SLX1737–282	17 37 47	–28 17 06	3.0	1.5 ± 0.22	7.7	0.92	0.28
	(17 37 36	–28 16 30	1.0)				
4 1E1740.7–2942	17 40 42	–29 43 30	1.0	11.6 ± 0.16	82.5	9.9	0.73
	(17 40 42	–29 43 15	0.5)				
5 1E1742.5–2859 = Sgr A*	17 42 29	–28 59 02	1.1	1.4 ± 0.13	5.2	0.62	0.19
6 A1742–294	17 42 56	–29 29 24	1.0	34.7 ± 0.14	147	17.6	(1.30)
	(17 42 54	–29 29 02	0.5)				
7 1E1743.1–2843	17 43 11	–28 42 43	1.0	6.9 ± 0.16	35.1	4.2	0.091
8 SLX1744–299	17 44 15	–29 59 46	1.0	7.8 ± 0.16	34.6	4.1	
9 GX3+1	17 44 49	–26 32 53	1.0	140.9 ± 0.50	540	64.6	
GX+0.2–0.2#				< 0.54	< 2.0	< 0.24	
4U1735–28#				< 0.83	< 3.1	< 0.37	

* Numbered as in Figs 1 and 2.

† Estimated from this work (1950 coordinates). Positions from Rev B analysis of Einstein data in parentheses.

‡ Counting rate from coarse telescope, except for source at Sgr A*, which was resolved from Source 3 only in the fine telescope. Statistical errors are one standard deviation in the count rate.

§ Flux in energy range 3–30 keV, derived from count rate using spectrum determined from SL2 data.

|| Apparent luminosity in energy range 3–30 keV, assuming a distance of 10 kpc.

¶ Expected counting rate based on Einstein observations and spectrum by WWGH, except that, on the basis of our observations, the same conversion for A1742–294 has been used as for the other sources. The count rate for this source in WWGH may have been affected by its position at the edge of the field; a prediction based on Seq. I 2358 would be 4.13 counts s^{-1} .

Upper limits are highest level in error box $+3\sigma$. Conversion to physical units assumes standard spectrum of WWGH.

Because it is large, the X-ray absorption is difficult to determine from the IPC data and we suggest that it is even higher than WWGH assumed in the absence of better information. Sanders *et al.*²⁵ have developed a model for the CO distribution in the Galaxy and reviewed the relationship between CO emission and H₂ mass. From their work, a column density to the galactic centre of 3×10^{23} atoms cm⁻² is expected from molecular hydrogen (the contribution from H I is small). This is five times the figure used by WWGH and, for a variety of underlying spectral shapes, such a change would alter the relationship between IPC counts s⁻¹ and SL2-XRT counts s⁻¹ by a factor of eight to ten.

These estimates of the H₂ density assume that elemental abundances are similar to those in the solar neighbourhood and may be wrong, perhaps by a factor as large as 10, because of metallicity gradients^{26,27}. The expected effect on X-rays is, however, insensitive to the abundance assumed, because above 0.5 keV the absorption is predominantly by the elements C-Fe, which are presumably well measured by the CO observations. Below 0.5 keV, photoelectric absorption in the interstellar medium (ISM) is effectively complete on any reasonable assumption. Changing the assumptions about the abundances of these elements relative to H alters the deduced H column density, but not the effect on an X-ray spectrum.

The surface brightness of the diffuse emission seen in the Einstein data is about 60% of that in the central peak of the SL2 data. This comparison uses the analysis and spectrum of WWGH, and the difference may be due to the same effects seen in the point source data. But, with the wider field of view of the SL2 instrument, it becomes apparent that the 33×15 arc min region in the Einstein data either is the central part of a much larger diffuse region 2° in diameter or is superimposed on a distinct component of that size. The total luminosity of the diffuse emission is thus very much larger than previously suspected. The Spartan observations are broadly consistent with this conclusion, although within the more limited region examined the extensive component is best modelled as a uniform background¹⁰.

The central part of the $\sim 2^\circ \times 80^\circ$ galactic ridge observed by Warwick *et al.*²⁸ presumably crosses our field of view, although they were not able to observe it in this region because of source confusion. The expected level is approximately four times lower than the diffuse emission we observe and it does not affect our conclusions.

Several authors^{1,28,29} have reviewed mechanisms which may give rise to diffuse, or apparently diffuse, X-ray emission in the galactic centre. We wish to point out that whatever the origin of the photons the density of interstellar matter is so high in this unique region of the Galaxy that X-ray scattering must be important. One type of scattering which must occur is from interstellar dust grains. Using the analysis of Mauche and Gorenstein³⁰, we expect that in the energy band of the Einstein IPC >50% of the photons observed through dust clouds with a visual extinction of 30 magnitudes, typical of the galactic centre, will have been scattered. The scattering angle θ_s is typically ~ 10 arc min, but the scattering usually occurs a small fraction (r/R) of the distance along the path to us and so the direction of arrival is not greatly affected ($\Delta\phi \approx (r/R)\theta_s$), although short time structure ($< (1.2 \text{ h})(\theta_s/10 \text{ arc min})^2 (r/10 \text{ pc})$) will be smeared out. The detailed shape of the image of a point source will be changed, but although the methods used by WWGH assumed the nominal point source response function, the effects are probably small and are unlikely to account for all the discrepancy between their results and those from SL2. The Rayleigh-Gans scattering cross-section varies approximately as E^{-2} , so scattering from dust is much less important in the SL2-XRT energy range.

Another possibly important effect is Compton scattering by electrons (bound or free) in the ISM. In the energy range of the SL2 instrument the Compton scattering cross-section is, to

a first approximation, simply the Thomson cross-section 6.6×10^{-25} cm² (corrections at low energy for electrons bound in H atoms and at high energies through the use of the Klein-Nishina cross-section exceed 20% only outside the range 5–45 keV). Thus if the typical H column density is indeed 3×10^{23} atoms cm⁻², $\sim 20\%$ of photons escaping the region will have been scattered. This is insufficient to explain the whole of the diffuse emission (which, at energies >4.5 keV, is about 55% of the intensity of the point sources) but would be an important component. We assume here that the present level of point source activity is typical, because the larger scattering angles associated with Compton scattering give characteristic time delays of ~ 100 yr.

If the metallicity in the galactic centre is high, so that molecular cloud observations overestimate the H column, then although X-ray absorption would be little affected, as discussed above, Compton scattering (mainly from electrons in H₂ and He) would be a smaller effect. This may provide a new probe into the H₂ and He components of the ISM, which are otherwise very difficult to detect.

In conclusion, our finding that the X-ray luminosity from the region of the galactic nucleus is higher than previously reported is probably because the interstellar absorption has been underestimated. Nevertheless, despite the violent and energetic processes seen in radio observations, the 'power house' at the nucleus of the Galaxy is somewhat puny at hard X-ray energies, compared with other sources in the region and in particular with the remarkable 1E1740.7–2942. More than a third of the 4.25–15 keV X-ray emission comes from a diffuse component much more luminous and extensive than previously reported. Further work to investigate the spectra and variability of the sources described here is now in progress. An X-ray burst was detected from the region and details of this too will be reported separately.

We are grateful to NASA for the opportunity to fly the XRT instrument on the Spacelab 2 mission, to SERC for support and to all the many people who made the mission a success. We thank members of the Centre for Astrophysics for help with Einstein data. Data analysis was carried out using the facilities of the Starlink network.

Received 13 August; accepted 1 October 1987.

1. Watson, M. G., Willingale, R., Grindlay, J. E. & Hertz, P. *Astrophys. J.* **250**, 142–154 (1981).
2. Proctor, R. J., Skinner, G. K. & Willmore, A. P. *Mon. Not. R. astr. Soc.* **185**, 745–754 (1978).
3. Kellogg, E., Gursky, H., Murray, S., Tananbaum, H. & Giacconi, R. *Astrophys. J.* **169**, L99–L103 (1971).
4. Cruddace, R. G. *et al. Astrophys. J.* **222**, L95–L98 (1978).
5. Skinner, G. K. *Proc. R. Soc. A* **366**, 345–355 (1979).
6. Jernigan, J. G. *et al. Nature* **272**, 701–704 (1978).
7. Forman, W. *et al. Astrophys. J. Suppl.* **38**, 357–412 (1978).
8. Eyles, C. J., Skinner, G. K., Willmore, A. P. & Rosenberg, F. D. *Nature* **257**, 291–293 (1975).
9. Wilson, A. M., Carpenter, G. F., Eyles, C. J., Skinner, G. K. & Willmore, A. P. *Astrophys. J.* **215**, L111–L115 (1977).
10. Kawai, N. *et al. Astrophys. J.* (submitted).
11. Willmore, A. P., Skinner, G. K., Eyles, C. J. & Ramsey, B. *Nucl. Instrum. Meth.* **221**, 284–287 (1984).
12. Skinner, G. K., Ponman, T. J., Hammersley, A. P. & Eyles, C. J. *Astrophys. Space Sci.* **136**, 337–349 (1987).
13. Hertz, P. & Grindlay, J. E. *Astrophys. J.* **278**, 137–149 (1984).
14. Makishima, K. *et al. Astrophys. J.* **247**, L23–L25 (1981).
15. Hertz, P. & Grindlay, J. E. *Astrophys. J.* **275**, 105–119 (1983).
16. Hertz, P. & Grindlay, J. E. *Astrophys. J.* **267**, L83–L87 (1983).
17. Dennis, B. R. *et al. Astrophys. J.* **236**, L49–L53 (1980).
18. Knight, F. K., Johnson, W. N. III, Kurfess, J. D. & Strickman, M. S. *Astrophys. J.* **290**, 557–567 (1985).
19. Matteson, J. L., in *The Galactic Center* (eds Riegler, G. R. & Blandford, R. D.) 109–121 (Am. Inst. Phys., New York, 1982).
20. Levine, A. M. *et al. Astrophys. J. Suppl. Ser.* **54**, 581–617 (1984).
21. Leventhal, M., MacCallum, C. J. & Stang, P. D. *Astrophys. J.* **225**, L11–L14 (1978).
22. Riegler, G. R. *et al. Astrophys. J.* **248**, L13–L16 (1981).
23. Riegler, G. R., Ling, J. C., Mahoney, W. A., Wheaton, W. A. & Jacobson, A. S. *Astrophys. J.* **294**, L13–L15 (1985).
24. Leventhal, M., MacCallum, C. J., Hutters, A. F. & Stang, P. D. *Astrophys. J.* **302**, 459–461 (1986).
25. Sanders, D. B., Solomon, P. M. & Scoville, N. Z. *Astrophys. J.* **276**, 182–203 (1984).
26. Blitz, L., Bloemen, J. B. G. M., Hermesen, W. & Bania, T. M. *Astr. Astrophys.* **143**, 267–273 (1985).
27. Bhat, C. L., Issa, M. R., Houston, B. P., Mayer, C. J. & Wolfendale, A. W. *Nature* **314**, 511–515 (1985).
28. Warwick, R. S., Turner, M. J. L., Watson, M. G. & Willingale, R. *Nature* **317**, 218–221 (1985).
29. Bhat, C. L., Kifune, T. & Wolfendale, A. W. *Nature* **318**, 267–269 (1985).
30. Mauche, C. W. & Gorenstein, P. *Astrophys. J.* **302**, 371–387 (1986).

Cometesimals in the inner Solar System

T. M. Donahue*, T. I. Gombosi* & B. R. Sandel†

* Space Physics Research Laboratory, Department of Atmospheric and Oceanic Science, The University of Michigan, Ann Arbor, Michigan 48109, USA

† Lunar and Planetary Laboratory, The University of Arizona, Tucson, Arizona 85721, USA

A large flux of small comets in the inner Solar System¹ might produce a recognizable Lyman- α signature near 1 AU (ref. 2). We have now examined spectra obtained by the ultraviolet spectrometer (UVS)³ on the Voyager 2 spacecraft between 1 and 2.5 AU and have found evidence for a very large number of 'cometesimals' with radii between a few metres and a few tens of metres in the neighbourhood of the Earth. The evidence consists of a component in the interplanetary Lyman- α radiation that decreases rapidly (between r^{-3} and r^{-4}) with heliocentric distance. The Lyman- α emission rate in a direction normal to the Sun-spacecraft line and downwind (the anti-apex direction) in the interstellar wind at 1 AU is 640 rayleighs, of which our analysis attributes 477 R to the interstellar medium (ISM) and 163 R to another source. Despite this result we propose that this source consists of comets of a different sort than those proposed by Frank *et al.*¹ and with a flux seven orders of magnitude smaller. We propose that these cometesimals are ice-coated, porous, low density refractory boulders that may be the building blocks of ordinary comet nuclei⁴. We show that the cometesimals required to produce the observed Lyman- α emission can also account for all the lunar craters with diameters between 200 m and 1,500 m produced during the past 3,200 million years at sites such as Mare Tranquillitatis.

In searching the UVS data obtained from Voyager 1 and 2 near 1 AU for a Lyman- α signature near 1 AU we found one sequence of 17 observations made between 1 AU to ~2.5 AU in which the field of view was always in about the same part of the sky, downwind in the ISM. The observations in that sequence that were obtained near 1 AU were made in a direction that was also perpendicular to the Sun-spacecraft line, but away from the geocorona and geotail. All were obtained at distances greater than 10^7 km from Earth, far outside the geocorona. We have corrected the Lyman- α emission rates for variations in solar Lyman- α (ref. 5) and normalized them to a solar flux of 5.43×10^{11} photons $\text{cm}^{-2} \text{s}^{-1} \text{\AA}^{-1}$, which was the representative value for late 1977 when the data were obtained. We have attempted to correct for the phase shift between the solar Lyman- α arriving at Earth and at the spacecraft. We estimate that the uncertainty in this correction is reflected in an uncertainty of $\pm 2.5\%$ in the data plotted in Fig. 1. We also plot five data points, those with vertical arrows, that were obtained in the same general part of the sky, but should contain somewhat more ISM contribution than the rest.

Figure 1 shows the Lyman- α variation expected for the ISM, normalized to 430 R at 3 AU, from a model of Thomas⁶. If, in addition to the ISM, there is a component in the atomic hydrogen local emission rate that varies as $r^{-\gamma}$, then an observation normal to the Sun-spacecraft axis at a distance r from the Sun will yield a column emission rate given by $r^{-(\gamma-1)}$. We also present curves showing the sum of the ISM and this second component of Lyman- α for values of γ between 3 and 5. The curves are normalized to an emission rate of 163 R in excess of the Lyman- α radiation from the downwind ISM at 1 AU. The uncertainty in corrected counting rate of $\pm 0.07 \text{ s}^{-1}$, or $\pm 13 \text{ R}$ is small enough that the small excess in Lyman- α between 2.3 and $2.7 \times 10^8 \text{ km}$ is probably significant.

We have calculated the rate at which water molecules are produced from clean ice as a function of heliocentric distance. The production rate varies as $r^{-2.3}$ and has a value of $1.17 \times$

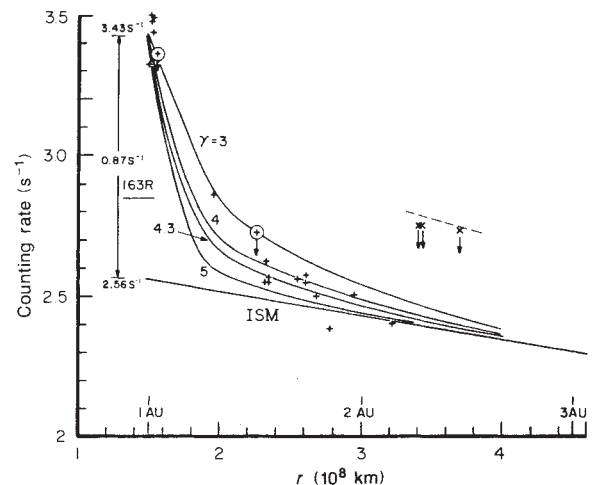


Fig. 1 Lyman- α apparent emission rate observed by the Voyager 2 UVS instrument, days 244–360, 1977. Curves are for ISM Lyman- α alone and ISM plus a contribution varying as $r^{-(\gamma-1)}$. Points with vertical arrows identify observations for which there is a higher ISM contribution by about 10% (dashed line segment).

$10^{-5} \text{ g cm}^{-2} \text{s}^{-1}$ ($3.90 \times 10^{17} \text{ cm}^{-2} \text{s}^{-1}$) at 1 AU. The curve for $\gamma = 4.3$ in Lyman- α emission rate corresponds to a variation in hydrogen density of $r^{-2.3}$. This will also be the radial dependence of the product of the water production rate and the hydrogen lifetime. The water production rate from the family of cometesimals at heliocentric distance r is given by

$$Q(r) = 4\pi a^2 FN = n/(2\tau) \quad (1)$$

where a is the cometesimal radius, N the spatial density of cometesimals, F the water production rate per unit surface area for one cometesimal, n is the hydrogen density and τ the lifetime of hydrogen against ionization ($2 \times 10^6 \text{ s}$ at 1 AU). Hence, the contribution to the emission rate at 1 AU, from cometesimals of radius a , is given by

$$4\pi I(a) = 8\pi r_0 j \tau F f(\gamma) N(a) a^2 \quad (2)$$

per metre of cometesimal radius, where j is the specific Lyman- α emission rate per atom of $2.58 \times 10^{-3} \text{ s}^{-1}$, and r_0 is $1.5 \times 10^{13} \text{ cm}$. For $\gamma = 4.3$ the value of $f(\gamma)$ is 0.72. The differential spatial density $N(a)$ is given by

$$N(a) = \Psi(a)/(2v_r) \quad (3)$$

where $\Psi(a)$ is the sum of inbound and outbound (inactive) cometesimal fluxes per metre of cometesimal radius. In this analysis we have assumed that N is constant and neglect the smearing produced by the motion of the hydrogen atoms. The emission rate is thus proportional to $a^2 \Psi(a)$.

There have been numerous observations of the interplanetary Lyman- α radiation between Earth and Venus. We know that the emission rate does not continue to rise as $r^{-(\gamma-1)}$ with $\gamma > 3.5$ inside 1 AU. We are led to conclude that, if cometesimals are the source of the interplanetary hydrogen, a significant fraction of them are losing their clean ice mantles around 1 AU and becoming inactive. Such objects might be porous hydrous silicate boulders with an outer shell of clean ice, whose radii range from tens of centimetres to ~100 m, such as those Gombosi and Houppis⁴ have argued may be the building blocks of larger comet nuclei. In this scenario these small cometesimals would be making their first swing through the inner solar system and therefore travelling with near parabolic velocity ($v_r = 30 \text{ km s}^{-1}$ at 1 AU).

We have attempted to determine a cometesimal flux that is at the same time consistent with lunar cratering rates and the observed Lyman- α production rate. A necessary condition is

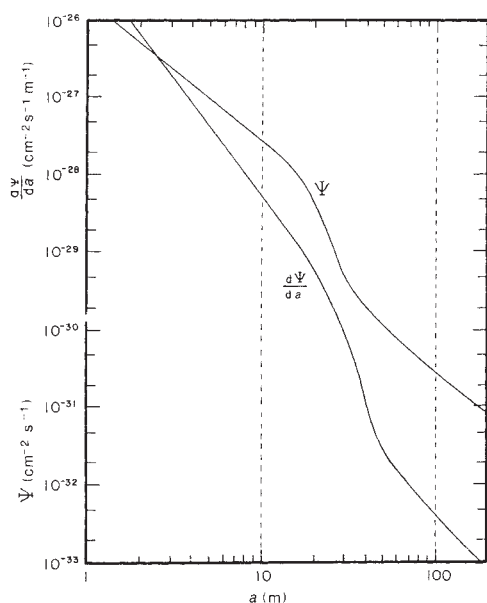


Fig. 2 Differential and integral cometsimal fluxes required to produce lunar crater densities at the Apollo 12 site 3.2×10^9 years old.

that the flux of cometsimals required to produce Lyman- α not produce a higher lunar crater density than the observed one. For the cumulative crater density we have used the lunar calibration curve and chronology presented by Neukum and Wise⁷. We use the cumulative crater density $C(D)$ for craters with diameters greater than D at the Apollo 12 site, 3.2×10^9 years old, to obtain a cratering rate. To relate impactor radius, velocity and density to crater diameter we use the recent experimental scaling laws obtained by Schmidt and Housen⁸,

$$\frac{D}{2} \left(\frac{\rho}{m} \right)^{1/3} = 0.8 \left(\frac{3.22 g a}{u^2} \right)^{-0.22} \quad (4)$$

where ρ is the density of the lunar surface, m the mass of the impactor, u the impact velocity, and g the lunar gravitational acceleration. For u we have averaged impact directions to obtain $u = 65 \text{ km s}^{-1}$. The choice of comet density is crucial. Supported by analysis of comet Halley⁹ that cometary densities lie between 0.1 cm^3 and 0.4 g cm^{-3} , we have chosen to use very small values for δ .

The cumulative flux for cometsimals with radii greater than required to reproduce the cratering data is

$$\Psi(a) = 4 \times 10^{-17} C(a) [\text{cm}^{-2} \text{ s}^{-1}] \quad (5)$$

and the differential flux is

$$\Psi(a) = \frac{d\Psi}{da} = 4 \times 10^{-17} \frac{dC}{dD} \frac{dD}{da} [\text{cm}^{-2} \text{ s}^{-1} \text{ m}^{-1}] \quad (6)$$

These limiting differential and integral fluxes are shown in Fig. 2, for $\delta = 0.1 \text{ g cm}^{-3}$. When the corresponding differential Lyman- α production rate from equation (6) is integrated down to $a = 3.2 \text{ m}$ (200-m diameter crater), the integral Lyman- α rate is 122 R, of which 114 R is produced by cometsimals with radii less than 40 m (Fig. 3). The crater density curves and corresponding cometsimal flux curves suggest that craters with diameters greater than 1,500 m are created by a different family of impactors than those with diameters less than 1,500 m. According to equation (4) a 1,500-m crater would be created by a cometsimal of 42-m radius. We propose to identify the generators of craters with $D < 1500 \text{ m}$ with our cometsimals and suggest that the flux of cometsimals cuts off when the cometsimal radius is large enough to make such a crater. To produce another 50 R of Lyman- α cometsimals with radii as small as 1.25 m would

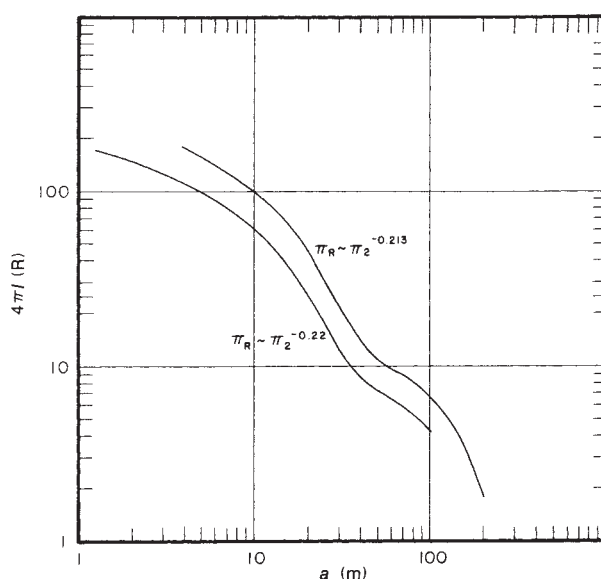


Fig. 3 Integral Lyman- α emission rate from cometsimals with radii greater than a for two assumptions regarding the scaling law for cratering.

be needed. On the other hand, a change of the exponent in the scaling law to 0.213 would permit cometsimals in the range from 4 m to 50 m to produce 163 R of Lyman- α . Hence, the intriguing conclusion is reached that the same family of cometsimals that accounts for the observed excess of interplanetary Lyman- α radiation above the ISM contribution at 1 AU will also produce all of the craters with radii less than 1,500 m that have appeared on the Moon during the past 3.2×10^9 years. Craters whose diameters are smaller than 200 m could be produced by cometsimals that have become inactive before reaching 1 AU.

We have also calculated the total amount of ice that would be lost by these cometsimals by the time they reach 1 AU. Cometsimals with perihelia between 1 AU and 0.5 AU would lose between 1.6 m and 2.3 m of ice between the Oort cloud and 1 AU. Hence, a low radius cutoff of a few metres for active comets at 1 AU is not unreasonable. Because of the strong dependence of Lyman- α production rate on cometsimal radius in this model, depending as it does on a $a^2 \Psi$, the failure of the Lyman- α emission rate to continue to vary as a high power of r inside 1 AU can be accounted for fairly naturally. The average distance between the smallest active cometsimals at 1 AU is only $\sim 10^{-2} \text{ AU}$ and it increases to $\sim 10^{-1} \text{ AU}$ for the largest. Thus, the implicit assumption in our treatment that the hydrogen distribution is homogeneous is easily satisfied. The interval between impacts on Earth for all cometsimals is about 80 years and for those with radii greater than 30 m is about 10^4 years.

We prefer the model presented here for the source of the extra component of interplanetary Lyman- α to the small comets of Frank *et al.*¹ for the reasons given by Donahue². To hold Lyman- α down to 163 R from the small comets of Frank *et al.*¹ the water production rate would need to be less than $10^{10} \text{ cm}^{-2} \text{ s}^{-1}$ which requires a dust mantle on the comets that would be at least 45 cm thick according to Fanale and Salvail¹⁰, but perhaps 12 km thick according to Horányi *et al.*¹¹. The integral flux of our cometsimals is 7 orders of magnitude smaller than that of the Frank *et al.*¹ small comets.

A comparison of observations of Lyman- α made near 1 AU from OGO-5 and at $\sim 1.35 \text{ AU}$ from Mariner 9 shows that OGO-5 observed about 110 R more Lyman- α than Mariner 9 when the direction of observation from each was perpendicular to the Sun-spacecraft axis and downwind in the ISM¹². From Fig. 1 the difference expected on the basis of the Voyager 2 observations and the $r^{-4.3}$ model is about 110 R. Although there are problems with comparing measurements made from different

spacecraft involving calibration and a possibility of contamination of the OGO-5 data from the geocorona, this comparison seems to support the results discussed in this paper.

We emphasize the many elements of uncertainty in this analysis. The data are sparse and scattered, and there is an unfortunate gap in the record between 1.033 AU to 1.3 AU. Thus, the radial dependence of Lyman- α is not well determined. (There will be an excellent opportunity to explore the entire region from 0.7 AU to 3 AU with the UVS on Galileo.) The treatment of hydrogen distribution in this paper neglects the smearing that is the consequence of cometary motion and the high velocity of the atoms produced by dissociation processes. Eventually, when and if a better data set becomes available, this effect should be taken into account. The r^{-2} dependence of $j(r)$ in equation (1) will always assure a rapid decrease in $4\pi I$ with r , however. The agreement with cratering data depends crucially on the density of the proposed cometsimals being very small. If their density should be as large as 0.25 g cm^{-3} , they would produce only 65 R of Lyman- α ($a > 1 \text{ m}$). Whether the scaling law even applies to such low density impactors is not clear. It is possible that the sensitivity of the UVS decreased and then stabilized when the spacecraft was about $2 \times 10^8 \text{ km}$ from the Sun. This seems unlikely because there was no apparent change in sensitivity during the first 15 days of the mission when the first seven data

points plotted in Fig. 1 were acquired. The model does require a surprisingly dramatic change in the Lyman- α productivity of these cometsimals very near 1 AU, but the ability of the model to explain the Lyman- α data and lunar crater density distribution is an appealing if not a compelling argument in its favour. Note that the cometsimals proposed here may have very clean icy surfaces and need not generate much dust. Hence, optical detection of even the largest may not be feasible. It would be worthwhile to try nevertheless.

We thank T. Ahrens and H. Masursky for useful information on cratering processes and lunar crater densities. This research was supported in part by the NSF and NASA.

Received 30 July; accepted 23 October 1987.

1. Frank, L. A., Sigwarth, J. B. & Craven, J. D. *Geophys. Res. Lett.* **13**, 307-310 (1986).
2. Donahue, T. M. *Geophys. Res. Lett.* **14**, 213-215 (1987).
3. Broadfoot, A. L. *et al. Space Sci. Rev.* **21**, 183-206 (1977).
4. Gombosi, T. I. & Houpsis, H. L. F. *Nature* **324**, 43-44 (1986).
5. Hinteregger, H. E. *Adv. Space Res.* **1**, 39-52 (1981).
6. Thomas, G. A. *Rev. Earth planet. Sci.* **6**, 173-204 (1978).
7. Neukum, G. & Wise, D. V. *Science* **194**, 1381-1387 (1976).
8. Schmidt, R. M. & Housen, K. R. *Eos* **67**, 1078-1079 (1986).
9. Mendis, D. A. *Proc. 20th ESLAB Symp. Exploration Halley's Comet* (ESA SP-250, 1986).
10. Fanale, F. P. & Salvail, J. R. *Icarus* **60**, 476-511 (1984).
11. Horányi, M. *et al. Astrophys. J.* **278**, 449-455 (1984).
12. Bohlin, R. C. *Astr. Astrophys.* **28**, 323-326 (1973).

Vapour pressure of amorphous H_2O ice and its astrophysical implications

Akira Kouchi

Institute of Low Temperature Science, Hokkaido University, Sapporo 060, Japan

Because an understanding of the physical properties of vapour-deposited amorphous H_2O , I_{as} , is important for the discussion of the evolution of the Solar System¹⁻³, they have been extensively studied⁴⁻⁹. In particular, the importance of vapour pressure of I_{as} for the production rate of H_2O gas from a new comet has been realized^{10,11}; but there has been no previous measurement of this. Here we present the results of experiments demonstrating that the vapour pressure of I_{as} is one or two orders of magnitude larger than that of crystalline H_2O and depends greatly on the condensation temperature, T_c , and the rate of condensation, R . We also discuss the evaporation of I_{as} in space.

A metal substrate in a vacuum chamber of $1 \times 10^{-6} \text{ Pa}$ (H_2O , $5 \times 10^{-7} \text{ Pa}$; $\text{N}_2 + \text{O}_2$, $5 \times 10^{-7} \text{ Pa}$) was cooled to T_c by continuous flow of liquid nitrogen. I_{as} was prepared by admitting H_2O vapour at room temperature into the vacuum system through a capillary tube of 1-mm diameter directed to a face of the substrate at a distance of 10 mm. The temperature of the substrate was controlled and measured with a Rh-Fe resistance thermometer mounted at the back of the substrate. Although the reading of the Rh-Fe sensor was calibrated in a separate experiment with Au-Fe7% Chromel thermocouples mounted at the surface of the substrate, we did not measure the temperature of the ice sample directly. The accuracy of the temperature measurement was $\pm 0.5 \text{ K}$ except for the temperature region near the phase transition of I_{as} to cubic ice, I_c , because the transition is exothermal. Under these conditions, the gas of H_2O monomer (revealed by the quadrupole mass spectrometer, QMS, Nichiden-Anelva AQS-360) immediately froze onto the substrate at a rate of $10^{-2-3} \text{ nm h}^{-1}$. The deposition rates were measured by the interferometry of a He-Ne laser beam with the thin ice film. After deposition, the substrate was warmed up at a rate of 1 K min^{-1} . The sublimation rates of H_2O were measured with the QMS, which was calibrated by the standard H_2O leak. Vapour pressures were calculated by assuming that the vaporiz-

ation coefficient, α_v , was close to unity¹²⁻¹⁴. The calculated vapour pressures were in good agreement with the reading of the calibrated ionization gauge ($\pm 5\%$) within $\pm 10\%$. The precision of the absolute value of vapour pressure was not so good because of the assumption that $\alpha_v = 1$. But the sensitivity and stability of the QMS is very good, so any different results obtained for amorphous ice are significant.

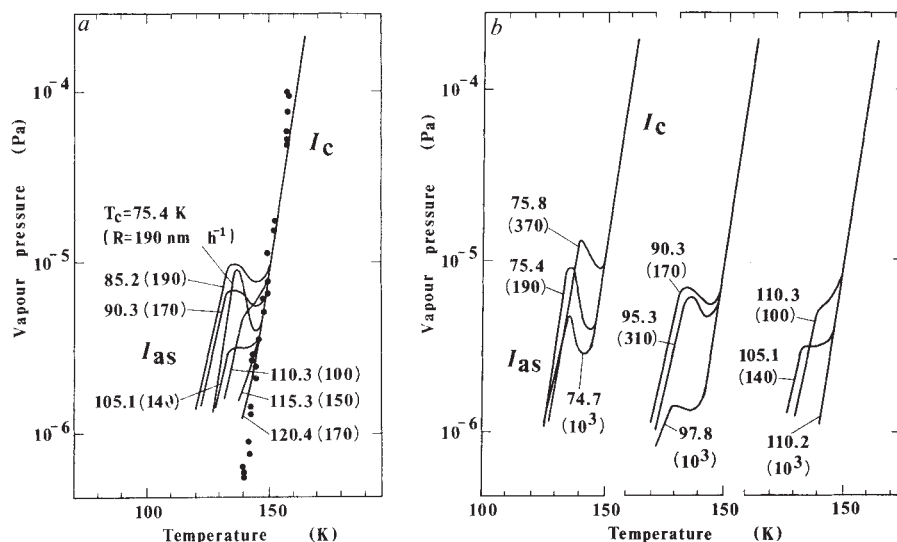
Figure 1 shows the temperature dependence of vapour pressure of H_2O ice produced at various T_c and R . It is apparent that the vapour-pressure curve is divided into two parts; the low-temperature part is considered to correspond to I_{as} ; the high-temperature part to I_c . The reasons are: first, previous studies show that I_{as} is formed by the precipitation of H_2O vapour onto the substrate at temperatures below $130\text{--}150 \text{ K}$; second, because I_{as} is energetically unstable compared with I_c , the vapour pressure of I_{as} must be higher than that of I_c . Indeed, current experimental results agree with this prediction and with the thermodynamic estimation by Léger *et al.*¹⁰. This is the first clear report of the direct measurement of the vapour pressure of I_{as} .

The vapour pressure of I_{as} is found to be one or two orders of magnitude larger than that of I_c , and to vary systematically as functions of T_c and R . In general, when T_c is low and when R is small, the difference in vapour pressure between I_{as} and I_c is large. On the other hand, with increasing T_c and R the vapour pressure of I_{as} approaches that of I_c . The temperature differences between the curves of I_{as} and that of I_c under constant pressure, range from 10 to 20 K. This difference is larger than that estimated by Léger *et al.* (3-4 K)¹⁰. Figure 1 also shows the tendency for the slope of I_{as} to increase with decreasing T_c and R . Current experimental results suggest that the difference in vapour pressure between different samples of I_{as} is sensitive to structural differences. Thus we conclude that the structure of I_{as} cannot be determined uniquely by temperature but is highly dependent on condensation temperature and condensation rate.

In Fig. 1a, the data of Bryson *et al.*¹⁴, whose T_c is rather high, 100 K, are also plotted. The two sets of data are fairly similar in the higher temperature part, but not in the low-temperature part. This comparison suggests (1) the accuracy of our measurement of vapour pressure is fairly good and (2) although they mentioned that the lower temperature part $< 150 \text{ K}$ corresponds to I_{as} , we do not know whether it is amorphous or not.

Figure 2 shows the relationship between T_c and the crystallization temperature of I_{as} , T_x . The T_x , defined by the peak of I_{as} ,

Fig. 1 The temperature dependence of vapour pressure of H₂O ice produced at various condensation temperatures, T_c (a) and condensation rates, R (b). Values without parentheses are T_c in kelvin; values in parentheses are R in nanometres per hour. I_{as} , amorphous H₂O; I_c , cubic ice. The data of Bryson *et al.*¹⁴ are shown by dots in (a).



increases with decreasing T_c . Thus I_{as} condensed at low temperatures is more stable up to higher temperatures. This shows that the crystallization temperature is not uniquely determined, but depends on T_c . Mayer and Pletzer⁸ also demonstrated that T_x depends irregularly on deposition time.

The properties of I_{as} shown in these experiments are reasonable characteristics of the nature of amorphous materials. Accordingly, if we discuss the physical and thermodynamic properties of I_{as} , we have to specify the conditions of condensation: condensation temperature⁵⁻⁹, condensation rate, the state of the H₂O vapour before deposition⁸, and thermal history^{7,8}.

We shall discuss the application of the experimental results to the sublimation process of I_{as} in space. All the previous discussion comes from the extrapolation of the vapour pressure of I_c ¹⁵. But these experiments demonstrated that the vapour pressure of I_{as} is higher than that of I_c as already shown. We must therefore discuss this process on the basis of present experimental results.

Figure 3 shows the schematic condensation diagram of H₂O ice in space. If crystalline ice is condensed at T_1 , the sublimation temperature at P_0 is T'_1 . On the other hand, I_{as} condensed at T_2 and T_3 sublimates at T'_2 and T'_3 , respectively. Accordingly, the sublimation temperature of I_{as} is lower than that of I_c even at the same pressure, P_0 , and is highly dependent on the condensation temperature and condensation rate. Because the condensation rate of these experiments is considerably higher than that in space, the vapour pressure of I_{as} condensed in space may have higher values than in the experiments. Note here that the temperature of H₂O vapour before deposition of the present experiments is rather high compared with that in space.

The vapour pressure of I_{as} is also important for the production rate of H₂O gas from a new comet. Mukai¹¹ suggested that the unexpectedly large production rate from comet Bowell (1980b)¹⁶ can be explained by: (1) the sublimation of heterogeneous icy grains with the vapour-pressure formula for I_{as} , which has a

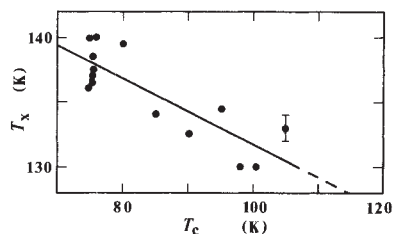


Fig. 2 The crystallization temperature T_x of I_{as} against condensation temperature, T_c . T_x is defined by the peak of vapour pressure of I_{as} (Fig. 1). We could not define T_x for $T_c > 105$ K.

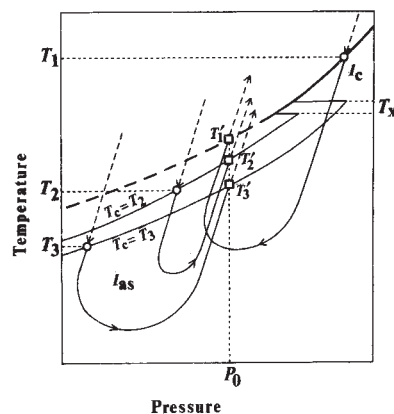


Fig. 3 A schematic illustration of condensation and sublimation of H₂O ice in space. The thick solid line shows the vapour pressure of I_c , where the condensation and sublimation of I_c occurs; the broken part is the low- T extrapolation, where only sublimation of I_c occurs. The thin solid lines labelled $T_c = T_2$ and $T_c = T_3$ are the vapour pressures of I_{as} condensed at T_2 and T_3 , respectively. Curves *a*, *b*, *c* show the supposed thermal history. The sublimation temperatures of ices at P_0 condensed at T_1 , T_2 and T_3 are T'_1 , T'_2 and T'_3 , respectively.

value one order of magnitude larger than that of I_c based on the estimation by Léger *et al.*¹⁰ and (2) a higher temperature than the computed value. The former possibility is qualitatively supported by these experimental results. Moreover, it seems sufficient to consider only the first of these two explanations, because the vapour pressure of I_{as} is two orders of magnitude larger than that of I_c as already shown. In general, the production rate of H₂O gas from a new comet before perihelion is larger than that after perihelion at the same heliocentric distance, because a new comet before perihelion is made of I_{as} , and after perihelion it is made of I_c .

I am indebted to Professors T. Kuroda, H. Hasegawa, J. M. Greenberg and T. Mukai for discussion, to Mr T. Segawa for the construction of a vacuum chamber, and to the late Professor T. Kobayashi for his encouragement. This work was partly supported by a grant-in-aid for fundamental research from the Japan Ministry of Education, and by the Research Aid of Inoue Foundation for Science.

Received 19 June; accepted 5 October 1987.

1. Klinger, J. *J. phys. Chem.* **87**, 4209-4214 (1983).
2. Greenberg, J. M. *et al. J. phys. Chem.* **87**, 4243-4260 (1983).
3. Seki, J. & Hasegawa, H. *Astrophys. Space Sci.* **94**, 177-189 (1983).

4. Hobbs, P. V. *Ice Physics* (Oxford University Press, 1974).
5. Narten, A. H. *et al. J. chem. Phys.* **64**, 1106–1121 (1976).
6. Sivakumar, T. C. *et al. J. chem. Phys.* **69**, 3468–3476 (1978).
7. Hagen, W. *et al. Chem. Phys.* **56**, 367–379 (1981).
8. Mayer, E. & Pletzer, R. *J. chem. Phys.* **80**, 2939–2952 (1984).
9. Mayer, E. & Pletzer, R. *Nature* **319**, 298–301 (1986).
10. Léger, A. *et al. Astr. Astrophys.* **117**, 164–169 (1983).
11. Mukai, T. *Astr. Astrophys.* **164**, 397–407 (1986).
12. Tschudin, K. *Helv. phys. Acta* **19**, 91–102 (1946).
13. Davy, J. G. & Somorjai, G. A. *J. chem. Phys.* **55**, 3624–3636 (1971).
14. Bryson, C. E. III *et al. J. chem. Engng Data* **19**, 107–110 (1974).
15. Washburn, E. W. in *International Critical Tables of Numerical Data* Vol. 3 (ed. Washburn, E. W.) 210–212 (National Research Council of the United States of America, 1928).
16. A'Hearn, M. F. *et al. Astr. J.* **89**, 579–591 (1984).

The glass–liquid transition of hyperquenched water

G. P. Johari*, Andreas Hallbrucker† & Erwin Mayer†

* Department of Materials Science and Engineering, McMaster University, Hamilton, Ontario L8S 4L7, Canada

† Institut für Anorganische und Analytische Chemie, Universität Innsbruck, A-6020 Innsbruck, Austria

Non-crystalline solid forms of water prepared by the usual two methods of vapour-deposition on a substrate^{1,2} and by compression of hexagonal ice in a piston cylinder apparatus at 77 K (refs 3, 4) do not seem to undergo a glass–liquid transition on heating^{2,5–7}. Neither of these two, or possibly three, non-crystalline forms seem to be interconvertible by a single thermodynamic path involving only temperature or pressure. Also, their molecular structures are thermodynamically discontinuous⁸, with the structure of bulk water above 273 K and of emulsified water in the supercooled state near 230 K (see also refs 2 and 9). It has been difficult, therefore, to resolve whether or not water supercools to a glassy state in a thermodynamically reversible manner. We now report the results of a calorimetric study of the glassy state of water obtained by rapid cooling or by hyperquenching, demonstrate the reversibility of its glass–liquid transition in the temperature range 113–148 K, and consider the implication to our understanding of its other non-crystalline solid forms.

Glassy, or vitrified, water was prepared by hyperquenching, at rates $>10^5$ K s⁻¹, of ≤ 3 μ m size droplets on a copper substrate held at 77 K (refs 10–12). Briefly, droplets made by means of an ultrasonic nebulizer were suspended as an aerosol in N₂ gas and allowed to enter a high vacuum cryostat through a 300 μ m aperture in the first, and 200 μ m in the second, procedure. Once inside, the droplets moved towards the copper substrate at supersonic speeds and deposited on it. After 1 h (300 μ m) and 2 h (200 μ m) of deposition, an ~ 2 –3-mm thick disk-shaped opaque layer of glassy water was removed from the plate and handled while submerged in liquid N₂. Several batches of glassy water were made. None contained more than 5% crystalline ice, as previously characterized by calorimetric and X-ray studies^{11,12}. A Perkin-Elmer differential scanning calorimeter model DSC-4 with TADS data acquisition was used, and the base-line for the empty cells was subtracted during each scan made with 15–27 mg of the glassy water. The many samples studied showed identical glass–transition behaviour.

Figure 1 shows the several steps of the differential calorimetric scans (DSC) of glassy water heated at a rate of 30 K min⁻¹. On initial heating from 113 K, a pronounced decrease in the heat output occurs up to 130 K (ref. 12). This appears as a broad exotherm. For samples annealed at 130 K for 1 h or more, this exotherm does not appear. Instead, the DSC scans of the annealed samples showed an S-shaped endotherm in the temperature range, 134–152 K, which is characteristic of the glass transition. Further heating showed a sharp exothermic peak, corresponding to crystallization of the liquid or fluid water to cubic ice. The ratio of the area of the crystallization exotherm

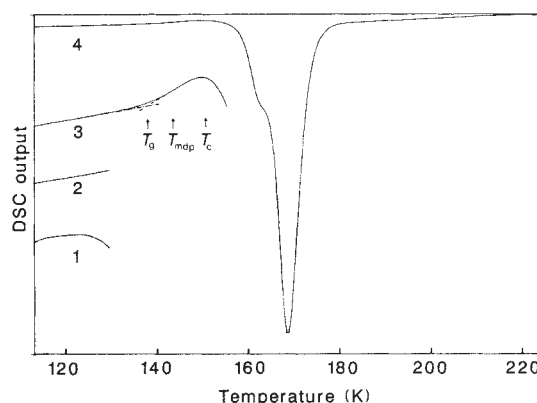


Fig. 1 The DSC scans of the hyperquenched glassy water (prepared by using a 300 μ m aperture) taken during heating at 30 K min⁻¹. Curve 1 is for a 17.7 mg sample heated to 130 K. Curve 2 is for the same sample, but after annealing for 95 min at 130 K, cooled to 103 K and heated to 130 K. Curve 3 is for the same sample which was cooled from 130 K to 103 K and again heated to 283 K. Curve 4 is the same as curve 3, but is plotted on a reduced scale (1/8th) to show the crystallization peak of glassy water to cubic ice. T_g is the glass transition temperature, T_{mdp} is the mid-point temperature, and T_c is the crystallization temperature, all corresponding to 30 K min⁻¹ heating rate. The temperature axis is not corrected for the thermal lag of the instrument.

to the area of the melting endotherm of hexagonal ice which was ultimately formed was constant, as before¹². This confirmed that crystallization of the glassy water to cubic ice did not occur during the annealing period at 130 K. The glass-transition temperature (T_g), taken as the onset point of the endotherm of the DSC scan, is 138 ± 1 K. The mid-point temperature of the glass-transition region is 144 ± 1 K, and the crystallization to cubic ice begins at 152 ± 1 K. After correction for the thermal lag of the instrument, the values are 136 ± 1 , 142 ± 1 and 150 ± 1 K respectively.

Similar observations for hyperquenched glassy water made using a 200 μ m aperture are shown in Fig. 2. The glass-transition endotherm in the DSC scans, taken after annealing the sample for 4 h, is more pronounced here than in Fig. 1. Figure 2 also shows the reversibility of the glass-transition region during thermal cycling of the hyperquenched sample to and from its relatively more fluid liquid state at 148 K. Clearly, glassy and liquid water follow an almost reversible path through a temperature range of 113–148 K, in which their structural states remain thermodynamically continuous and crystallization to cubic ice does not occur.

In the DSC scans, the structural relaxation time calculated for the heating rate of 30 K min⁻¹ is ~ 70 s at the onset temperature, or T_g , and ~ 7 s at the mid-point of the glass-transition endotherm. This implies that nucleation rate and/or crystal growth is too slow to detectably affect the glass–liquid transition of water. By assuming an exponential dependence of the structural relaxation time with reciprocal temperature in the narrow range of the glass–liquid transition, we calculate that the structural relaxation time of the H-bonded network of glassy water at its crystallization temperature of 151 K is 0.4 s. Thus a useful high-temperature limit and time limit for the handling of cryofixed biological materials in water hyperquenched to a glassy state is ~ 151 K and 0.4 s respectively.

From the approximate relaxation times given above, the 'activation' energy near T_g is $\sim 55 \pm 5$ kJ mol⁻¹. This is substantially less than that observed for SiO₂, silicates and other network glasses¹³, but is comparable with that of several hydrogen-bonded liquids¹⁴. This is evident also from the $\sim 12^\circ$ width (or more, if partially masked by crystallization) of the glass-transition region in Figs 1 and 2. This width is much greater for glassy water than for other substances, such as aqueous LiCl solutions⁵,

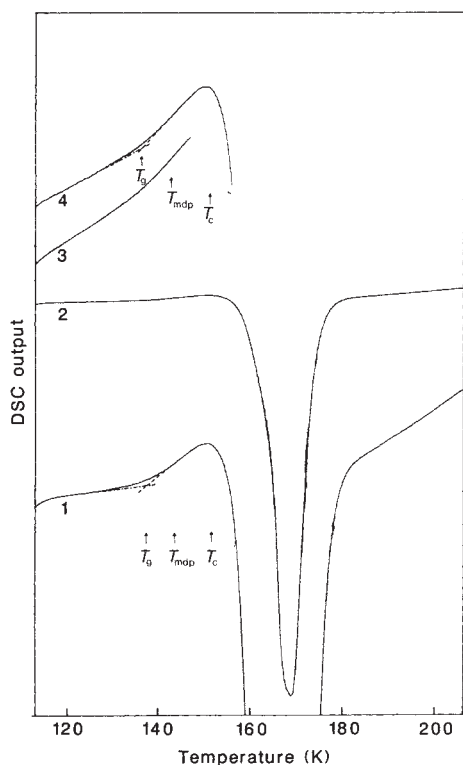


Fig. 2 The DSC scans of the hyperquenched glassy water prepared by using a 200 μm aperture. Curve 1 is for a 26.9 mg sample annealed at 130 K for 4 h, cooled to 103 K and heated. Curve 2 is the same as curve 1, but plotted on a reduced scale (1/8th). Curve 3 is for a 15.5 mg sample annealed at 130 K for 1.5 h and heated to 148 K. Curve 4 is for the same sample which was cooled immediately after curve 3 from 148 K to 103 K at a rate of 200 K min^{-1} while inside the calorimeter, then heated to 130 K, annealed for 1 h at 130 K, again cooled to 103 K, and finally heated for the DSC scan. The notations are the same as in Fig. 1. The heating rate was 30 K min^{-1} . The temperature axis is not corrected for the thermal lag of the instrument.

Se (ref. 15) or propanediol. Furthermore, the height of the glass-transition endotherm is much less than that observed for substances other than network type glasses¹³.

The main difficulties in observing a glass-liquid transition in finely powdered materials containing trapped and/or absorbed gases is that during heating, (1) their surface energy decreases as a result of sintering and (2) gas molecules are desorbed from their surface. Vapour-deposited and pressure-amorphized materials often show this type of behaviour. For glasses in general, a further difficulty is caused by the spontaneous enthalpy relaxation, which for a hyperquenched glass appears as an exotherm (Fig. 1) at $T \ll T_g$ and continues up to T_g . For water in particular, rapid crystallization to cubic ice creates an additional difficulty by masking the relatively slow and small increase in the heat capacity that occurs at its T_g . These effects masked the glass-transition endotherm in a previous study¹². Our procedure has removed these difficulties and revealed the glass transition in water and its thermodynamic reversibility.

These results are significant for understanding several aspects of amorphous ice and water, namely (1) the structural transition of the supercooled emulsified water, indicated by an approach towards a λ -type anomaly near 230 K (ref. 16), which makes it difficult to reconcile the structure of non-crystalline solid with that of liquid water⁸, (2) the thermodynamic continuity of the structural states between water and its non-crystalline solids, if the postulated λ -type anomaly was absent⁸ and (3) the polymorphism of amorphous solid water within the restrictions of hydrogen-bonded networks^{8,17,18}. To help resolve some of these

issues, we are currently studying the heat capacity of hyperquenched water and its transformation to other non-crystalline structures on compression at 77 K.

Financial support by the Forschungsförderungsfonds of Austria is gratefully acknowledged.

Received 22 June; accepted 2 October 1987.

- Burton, E. F. & Oliver, W. F. *Proc. R. Soc. A* **153**, 166 (1935).
- Sceats, M. G. & Rice, S. A. in *Water—A Comprehensive Treatise* (ed. Franks, F.) Ch. 2 (Plenum, New York, 1982).
- Mishima, O., Calvert, L. D. & Whalley, E. *Nature* **310**, 393–395 (1984); **314**, 76–78 (1985).
- Johari, G. P. & Jones, S. J. *Phil. Mag.* **54B**, 311–315 (1986).
- MacFarlane, D. R. & Angell, C. A. *J. phys. Chem.* **88**, 759–762 (1984).
- Handa, Y. P., Mishima, O. & Whalley, E. *J. chem. Phys.* **84**, 2766–2770 (1986).
- Mayer, E. & Pletzer, R. *J. chem. Phys.* **80**, 2939–2952 (1984).
- Johari, G. P. *Phil. Mag.* **35**, 1077–1090 (1977).
- Angell, C. A. in *Water—A Comprehensive Treatise* (ed. Franks, F.) Ch. 1 (Plenum, New York, 1982).
- Mayer, E. *J. appl. Phys.* **58**, 663–667 (1985); *J. phys. Chem.* **89**, 3474–3477 (1985).
- Mayer, E. *J. phys. Chem.* **90**, 4455–4461 (1986).
- Hallbrucker, A. & Mayer, E. *J. phys. Chem.* **91**, 503–505 (1987).
- Wong, J. & Angell, C. A. in *Glass: Structure by Spectroscopy* Ch. 1 (Dekker, New York, 1976).
- Johari, G. P. & Goldstein, M. *J. chem. Phys.* **53**, 4245–4252 (1971).
- Stephens, R. B. *J. Non-Cryst. Solids* **20**, 75–81 (1976).
- Angell, C. A., Shuppert, J. & Tucker, J. C. *J. phys. Chem.* **77**, 3092–3097 (1973).
- Maddox, J. *Nature* **326**, 823 (1987).
- Speedy, R. J. *J. phys. Chem.* **86**, 982–991 (1982).

Sonoluminescence from non-aqueous liquids

Kenneth S. Suslick & Edward B. Flint

School of Chemical Sciences, University of Illinois at Urbana-Champaign, 505 S. Mathews Avenue, Urbana, Illinois 61801, USA

Our understanding of the chemical effects of high-intensity ultrasonic irradiation of liquids is still quite limited. It is generally accepted that sonochemistry results from acoustic cavitation: the creation, growth, and implosive collapse of bubbles in ultrasonically irradiated liquids¹. The mechanism of sonoluminescence in aqueous systems has been a matter of some dispute; recent discussions have suggested at least three possible origins: black-body emission², chemiluminescence from radical recombination³, and electric discharge⁴. Few studies of non-aqueous sonoluminescence, however, have been conducted^{5–7}. We present here the first spectrally resolved sonoluminescence spectra from hydrocarbon and halocarbon liquids. These spectra originate unambiguously from excited-state molecules created during acoustic cavitation. These high-energy species probably result from the recombination of radical and atomic species generated during the high temperatures and pressures of cavitation.

The sonoluminescence spectrum of dodecane originates from excited state C_2 , specifically from the $d^3\Pi_g \rightarrow a^3\Pi_u$ transition—the so-called Swan band (Fig. 1). The four bands at 435, 465, 510 and 550 nm correspond to $\Delta v = +2, +1, 0$, and -1 of the vibrational manifold, respectively. The sonoluminescence spectra of mesitylene, 4-heptanone and *n*-butylcyanide are qualitatively similar to that of dodecane, but with lower intensities and slight changes in the relative intensities of the vibrational bands.

Swan band chemiluminescence is seen from hydrocarbons in a wide variety of conditions, including flames⁸, shock tubes^{9,10}, shock pyrolysis¹¹, plasmas¹², laser ablation¹³, and low pressure discharges¹⁴. Grebe and Homann¹⁴ in their study of the gas-phase reactions of acetylene with oxygen and hydrogen atoms ($C_2H_2/O/H$) in a low-pressure discharge flow reactor, provided compelling evidence that one reaction responsible for the generation of $C_2(d^3\Pi_g)$ was



For several reasons, this is also a plausible step in the sonochemical formation of $C_2(d^3\Pi_g)$. First, the pressures and

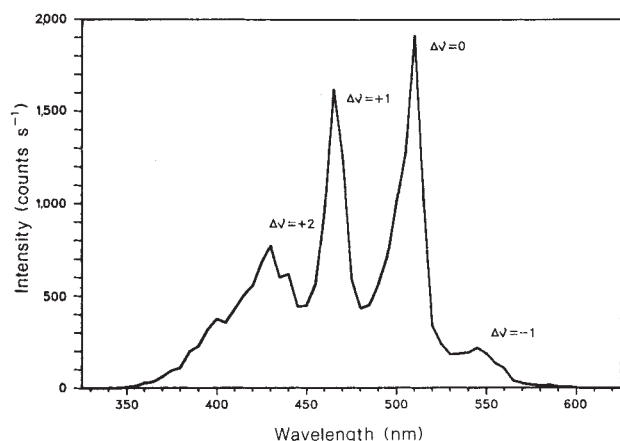


Fig. 1 The sonoluminescence spectrum of dodecane under argon. Emission is from excited state C_2 ($d^3\Pi_g \rightarrow a^3\Pi_u$). The cell temperature was -4°C ; vapour pressure at this temperature²⁵ is 0.006 torr.

Methods. All chemicals and gases were purified by standard methods²⁶. The irradiation cell has been described in detail elsewhere²⁷; the source of ultrasound was a direct immersion titanium horn operating at 20 kHz, which produced a 1 cm diameter collimated beam (far field region) with intensity of $\approx 25 \text{ W cm}^{-2}$ at the horn surface. Solutions were sparged with Ar during irradiation. Emitted light was collimated, passed into 0.25 m monochromator with 2.0 cm slits, and detected with a RCA 1P28 photomultiplier tube connected to a boxcar integrator. Spectra were collected at 5 nm increments and were not corrected for detector response.

temperatures achieved during acoustic cavitation are similar to those reached in shock tubes and plasmas. The effective temperature reached during cavitation in alkane solvents was recently determined¹⁵ to be $\approx 5,200 \text{ K}$ in the gas phase and $\approx 1,900 \text{ K}$ in the liquid region surrounding the collapsed bubble, when the total vapour pressure is 5 torr. Since the efficacy of cavitation collapse is inversely dependent on the total vapour pressure¹⁶ the effective temperatures generated in these sonoluminescent systems (whose vapour pressures are much less than 5 torr) must be even higher. Second, carbon-carbon bond cleavage of alkanes and the formation of radicals from non-aqueous liquids during ultrasonic irradiation has been documented¹⁶. The sonolysis of even *n*-alkanes generates a wide variety of homolytic products via a radical chain pathway. Atomic carbon and CH_2 can be created through such mechanisms. Indeed, the final products observed¹⁶ from alkane sonolysis include large amounts of H_2 , CH_4 and C_2H_2 , consistent with the observed sonoluminescence. Third, other techniques which generate comparable temperatures and pressures on a macroscopic scale also produce Swan band emission. For example, the shock tube pyrolyses of benzene⁹ and acetylene¹⁶ produced chemiluminescence spectra that could be assigned to $C_2(d^3\Pi_g)$, and when liquid benzene was shock compressed to between 24 to 63 GPa, emission from the Swan bands of C_2 was also observed¹¹.

The sonoluminescence spectrum of tetrachloroethylene is shown in Fig. 2. This can also be assigned to the C_2 ($d^3\Pi_g \rightarrow a^3\Pi_u$) Swan transition. The change in the relative vibrational band intensities and the strong diminution of the $\Delta v = +2$ band indicates a lower internal energy in C_2^* for tetrachloroethylene than for dodecane. This is consistent with the decrease in cavitation heating observed with increased total vapour pressure¹⁶. Several systems involving organic halides produce C_2^* chemiluminescence, including reactions with hydrogen atoms at low pressure¹⁷, with sodium atoms in a heat-pipe-oven reactor¹⁸, and with N_2O and sodium¹⁹. The formation of C_2^* is attributed to the reaction shown in equation 2. The abstraction of a halogen atom by another atom (H or

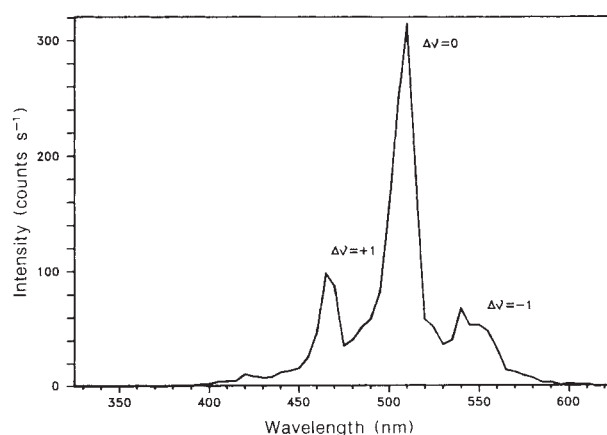


Fig. 2 The sonoluminescence spectrum of tetrachloroethylene under argon. Emission is from excited state C_2 ($d^3\Pi_g \rightarrow a^3\Pi_u$). The cell temperature was -16°C ; vapour pressure at this temperature²⁵ is 1.3 torr.

Na) generates the species that eventually luminesce. During ultrasonic irradiation, however, these radicals are formed by the high temperatures of the cavitation event. The sonochemistry of haloalkanes is little explored, although the sonolysis of chloroform to produce radicals and carbenes has been recently reported^{20,21}.



Figure 3 shows the sonoluminescence spectrum and the ultraviolet-visible absorption spectrum of nitroethane. The intense absorption of nitroethane at wavelengths below 375 nm prevents detection of any spectral features in the ultraviolet. This sonoluminescence emission can be assigned²² to the NO emission bands from the $\text{B}^2\Pi \rightarrow \text{X}^2\Pi$ transition (the so-called β bands), with $\Delta v \geq 12$. The spectrum is *not* consistent with the chemiluminescence observed²³ from NO_2^* , which is a broad emission from 400 to 1,200 nm with a maximum at about 650 nm. The reaction of carbon atoms with NO_2 (equation 3) has been studied in a low-pressure gas discharge reactor²⁴, and was identified as the only plausible mechanism for the formation of $\text{NO}(\beta)$. This mechanism is consistent with those proposed for C_2^* sonoluminescence.



These sonoluminescence spectra of non-aqueous liquids demon-

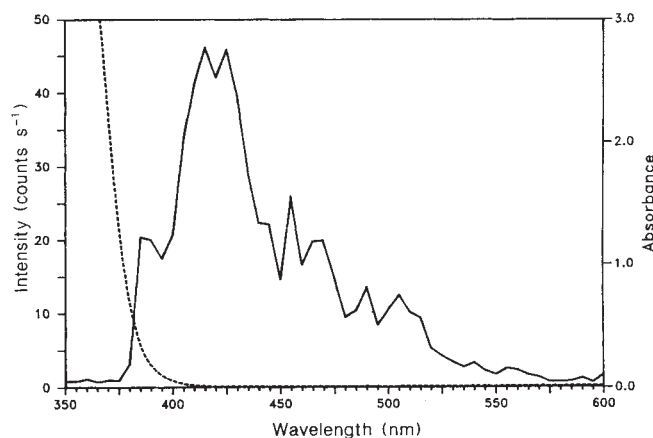


Fig. 3 The sonoluminescence spectrum of nitroethane under argon, scale at left. —, Emission from excited state NO ($\text{B}^2\Pi \rightarrow \text{X}^2\Pi$). The cell temperature was -19°C ; vapour pressure at this temperature²⁵ is 1.1 torr. - - - -, The ultraviolet-visible spectrum of nitroethane, 1 cm path length, absorption scale at right.

strate that ultrasound is a powerful chemical initiator and that significant chemistry occurs during ultrasonic irradiation even in the absence of reactive solutes. The similarity between sonoluminescence and the chemiluminescence seen with other high energy techniques (such as flames, plasmas, and shock tubes) indicates that similar chemistry is occurring during the ultrasonic irradiation of liquids.

We thank Dr A. Scheeline for assistance in instrument design, and the NSF for its support. K.S.S. gratefully acknowledges receipt of an NIH Research Career Development Award and of a Sloan Foundation Research Fellowship.

Received 5 June; accepted 24 September 1987.

1. Suslick, K. S. *Adv. organomet. Chem.* **25**, 73-119 (1986); *Modern Synthetic Methods* **4**, 1-60 (1986) (ed.) *Ultrasound: Its Chemical, Physical, and Biological Effects* (VCH, New York, 1987).
2. Walton, A. J. & Reynolds, G. T. *Adv. Phys.* **33**, 595-660 (1984).
3. Verrall, R. E. & Sehgal, C. *Ultrasonics* **25**, 29-30 (1987).
4. Margulis, M. A. *Ultrasonics* **23**, 157-169 (1985).
5. Chambers, L. *J. chem. Phys.* **5**, 290-292 (1937).
6. Jarman, P. *Proc. phys. Soc.* **73**, 628-640 (1959).

7. Golubnichii, P. I., Goncharov, V. D. & Protopopov, Kh. V. *Sov. Phys. Acoust.* **16**, 323-326 (1971).
8. Gaydon, A. G. *Spectroscopy of Flames* 2nd edn (Chapman and Hall, New York, 1974).
9. Kanoo, S. K. *Spectroscopy Lett.* **14**, 239-248 (1981).
10. Cooper, D. M. & Nicholls, R. W. *Proc. 10th Int. Shock Tube Symp.* (ed. Kamimoto, G.) 696-703 (Shock Tube Research Society, Tokyo, 1975).
11. Nicol, M., Johnson, M. L. & Holmes, N. C. *Physica* **139/140B**, 582-586 (1986).
12. Heinrich, G., Nickel, M., Mazurkiewicz, M. & Avni, R. *Spectrochimica Acta* **33B**, 635-647 (1978).
13. Anselmetti, M., Smith, R. S., Daykin, E. & Dimauro, L. F. *Chem. Phys. Lett.* **134**, 444-449 (1987).
14. Grebe, J. & Homann, K. H. *Ber. Bunsenges. phys. Chem.* **86**, 587-597 (1982).
15. Suslick, K. S., Hammerton, D. A. & Cline, R. E. Jr *J. Am. chem. Soc.* **108**, 5641-5642 (1986).
16. Suslick, K. S., Gawienowski, J. W., Schubert, P. F. & Wang, H. H. *J. phys. Chem.* **87**, 2299-2301 (1983); *Ultrasonics* **22**, 33-36 (1984).
17. Arnold, S. J., Kimbell, G. H. & Snelling, D. R. *Can. J. Chem.* **53**, 2419-2425 (1975).
18. Wang, X. Li, X. & Lou, N. *Chem. Phys.* **61**, 25-33 (1981).
19. Luria, M., Eckstrom, D. J. & Benson, S. W. *J. chem. Phys.* **64**, 3103-3110 (1976).
20. Suslick, K. S. & Schubert, P. F. *J. Am. chem. Soc.* **105**, 6042-6044 (1983).
21. Henglein, A. & Fischer, C. H. *Ber. Bunsenges. Phys. Chem.* **88**, 1196-1199 (1984).
22. Young, R. A. & Sharpless, R. L. *J. chem. Phys.* **39**, 1071-1102 (1963).
23. Sutoh, M., Morioka, Y. & Nakamura, M. *J. chem. Phys.* **72**, 20-24 (1980).
24. Dorthé, G. *et al. J. chem. Phys.* **82**, 2313-2320 (1985).
25. Riddick, J. A. & Bunger, W. G. *Techniques of Chemistry* Vol. 2 (Wiley, New York, 1970).
26. Perrin, D. D., Armarego, W. L. F. & Perrin, D. R. *Purification of Laboratory Chemicals* 2nd edn (Pergamon, New York, 1980).
27. Suslick, K. S. & Flint, E. B. in *New Developments in the Synthesis, Manipulation and Characterization of Organometallic Compounds* (American Chemical Society, Washington, DC, in the press).

Stable Ga-Mg-Zn quasi-periodic crystals with pentagonal dodecahedral solidification morphology

Wataru Ohashi & Frans Spaepen

Division of Applied Sciences, Harvard University, Cambridge, Massachusetts 02138, USA

The discovery in 1984 by Shechtman and co-workers of an Al-Mn alloy phase that exhibited a sharply peaked diffraction pattern with icosahedral point symmetry ($m\bar{3}5$) was a major development in crystallography¹. It was the first demonstration that quasi-periodic structures with fivefold symmetry axes, which had been described in the mathematical literature and whose sharp reciprocal lattice peaks could be indexed with two sets of incommensurate basis vectors, did actually occur in nature. This discovery has been followed by intense experimental and theoretical work; many more icosahedral and decagonal crystals have been discovered, and the theory describing the quasi-periodic lattices and their defects was greatly developed²⁻⁴. With one exception, all quasi-periodic crystals that have been discovered until now are metastable; they are produced by non-equilibrium methods, such as melt quenching, ion-beam mixing and sputtering, and upon heating they transform to phases with a periodic crystal structure. The only stable quasi-periodic crystal that was known is found in the Al-Li-Cu system, and has a striking rhombic triacontahedral solidification morphology⁵⁻⁷. We report here the discovery in the Ga-Mg-Zn system of a second stable quasi-periodic crystal with a pentagonal dodecahedral solidification morphology.

Villars *et al.*⁸ and Chen and Inoue⁹ had reported that quasi-periodic crystals could be formed in the Ga-Mg-Zn system by rapid solidification. We prepared an icosahedral phase of composition $\text{Ga}_{1.0}\text{Mg}_{1.8}\text{Zn}_{2.1}$ by melt spinning. Figure 1 shows the characteristic electron diffraction pattern taken along the fivefold axis of one of the crystals. We discovered that this phase was stable by heating it in the differential scanning calorimeter. Figure 2 shows the result: no exothermal peaks, characteristic of the non-equilibrium transformation to a more stable phase, were observed; the endothermic peaks correspond to peritectic melting. The melting phenomena above 680 K were confirmed by direct observation. Annealing of the icosahedral phase for 52 h at 638 K did not induce any transformations and left the electron diffraction pattern unchanged.

Based on this evidence for the thermodynamic stability of this icosahedral phase we concluded that it should be possible



Fig. 1 Electron diffraction pattern from a $\text{Ga}_{1.0}\text{Mg}_{1.8}\text{Zn}_{2.1}$ icosahedral crystal in a melt-spun ribbon.

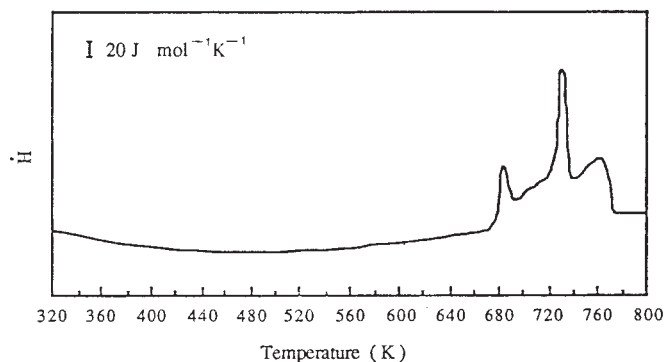


Fig. 2 Differential scanning calorimeter signal from the icosahedral $\text{Ga}_{1.0}\text{Mg}_{1.8}\text{Zn}_{2.1}$ phase produced by melt spinning. Only melting reactions are observed.

to obtain large crystals by slow cooling from the melt. We prepared several alloys that were solidified in a graphite mould under a helium-hydrogen atmosphere at a cooling rate of about 3 K min^{-1} . Several crystals of the type shown in Fig. 3 were found in a shrinkage cavity at the top surface of an alloy with average composition $\text{Ga}_{1.0}\text{Mg}_{2.1}\text{Zn}_{3.0}$. The growth morphology of these crystals is pentagonal dodecahedral (not pyritohedral as is some-

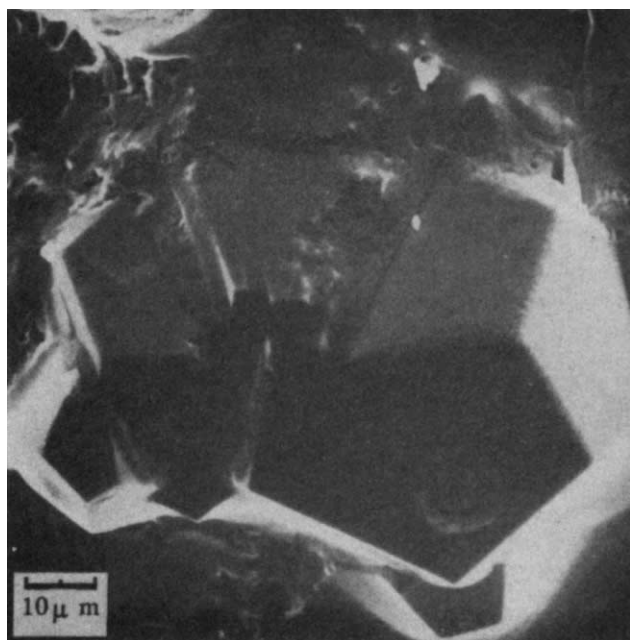


Fig. 3 Scanning electron micrograph of icosahedral crystals with pentagonal dodecahedral growth morphology found in a shrinkage cavity of a slowly cooled $\text{Ga}_{1.0}\text{Mg}_{2.1}\text{Zn}_{3.0}$ ingot.

times found for cubic crystals¹⁰); all the angles between edges on the same face were found to be 108° by matching them with the edges of an appropriate projection of a regular pentagon. The largest crystal found so far had a diameter of $\sim 100 \mu\text{m}$.

Some of these crystals were extracted by crushing the ingot and were prepared for transmission electron microscopy by ion milling. Figure 4 shows the selected area diffraction pattern along the macroscopically observed fivefold axis. The major peaks in the pattern have the characteristic tenfold symmetry. Some of the minor peaks are shifted slightly from their ideal positions (those of Fig. 1). Except for a few faint additional reflections, the pattern of Fig. 4 is identical to the one found by Lubensky *et al.* for a rapidly solidified icosahedral Al-Cr-Ru alloy¹¹. They attributed the distortions of the pattern to phason strains in the structure.

The ingots also contained the cubic Frank-Kasper phase that had been identified in this system¹². Its composition, GaMg_2Zn_3 ,

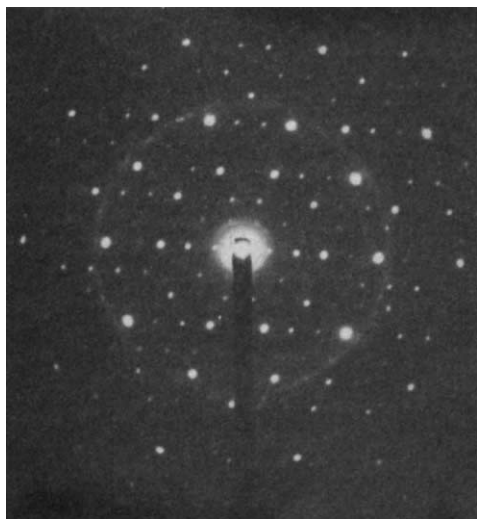


Fig. 4 Electron diffraction pattern from an icosahedral crystal formed in the same way as those of Fig. 3.

is close to that of the icosahedral phase. Its diffraction patterns were identical to those found by Guyot and Audier¹³ for the α -(Al-Fe-Si) phase, and by Audier *et al.*¹⁴ for the cubic (Al, Zn)₄₉Mg₃₂ phase, and could easily be distinguished from the icosahedral ones, even in pseudo-fivefold directions such as [035] and [01 τ] (τ is the golden ratio).

This discovery of a second stable quasi-periodic crystal will be of interest for diffraction studies of the structure and defects of these phases. It is somewhat surprising, for example, that the slowly cooled crystals have a more distorted diffraction pattern than the rapidly solidified ones. Their low melting temperature and the chemical behaviour of the alloys makes the crystals quite easy to prepare. Finally, the occurrence of different growth morphologies in icosahedral crystals is a new observation that needs further theoretical explanation.

We thank David Nelson and Subir Sachdev for many conversations emphasizing the importance of Frank-Kasper phases in identifying new quasicrystals and for informing us of Villars's identification of many Frank-Kasper compounds, including GaMg_2Zn_3 , tabulated in ref. 12. We thank Stephane Ebalard for help with the preparation of the alloys. This work has been supported by the National Science Foundation through the Harvard Materials Research Laboratory. W.O. acknowledges support by a scholarship from the Nippon Steel Corporation.

Received 14 July; accepted 2 October 1987.

1. Shechtman, D., Blech, I., Gratias, D. & Cahn, J. W. *Phys. Rev. Lett.* **53**, 1951-1953 (1984).
2. Nelson, D. R. & Halperin, B. I. *Science* **229**, 233-238 (1985).
3. Nelson, D. R. *Scient. Am.* **255**, 42-51 (1986).
4. Henley, C. L. *Commun. Condensed Matter Phys.* **13**, 59-117 (1987).
5. Dubost, B., Lang, J. M., Tanaka, M., Sainfort, P. & Audier, M. *Nature* **324**, 48-50 (1986).
6. Guyot, P. *Nature* **326**, 640-641 (1987).
7. Gayle, F. W. J. *Mater. Res.* **2**, 1-4 (1987).
8. Villars, P., Phillips, J. C. & Chen, H. S. *Phys. Rev. Lett.* **57**, 3085-3088 (1986).
9. Chen, H. S. & Inoue, A. *Scr. Metall.* **22**, 527-530 (1987).
10. Buerger, M. J. *Elementary Crystallography* 161 (MIT, Cambridge, Massachusetts, 1978).
11. Lubensky, T. C., Socolar, J. E. S., Steinhardt, P. J., Bancel, P. A. & Heinley, P. A. *Phys. Rev. Lett.* **57**, 1440-1443 (1986).
12. Villars, P. & Calvert, L. D. *Person's Handbook of Crystallographic Data for Intermetallic Phases* Vol. 3, 2277 (American Society for Metals, Metals Park, Ohio, 1985).
13. Guyot, P. & Audier, M. *Phil. Mag.* **B52**, L15-L19 (1985).
14. Audier, M., Sainfort, P. & Dubost, B. *Phil. Mag.* **B54**, L105-L111 (1986).

Ceramics ductile at low temperature

J. Karch, R. Birringer & H. Gleiter

University of Saarbrücken, FB 12.1, Bau 2, D-6600 Saarbrücken, FRG

It is generally recognized that ceramics frequently offer advantages over metals in their use, such as chemical resistivity, hardness, wear resistance, high melting temperature, low density and low price. However, the use of ceramics has so far been hampered by their brittleness at low temperature. Here we report observations that could open a way to remove these limitations. We observed that conventionally brittle ceramics became ductile permitting large ($\sim 100\%$) plastic deformations at low temperature if a polycrystalline ceramic was generated with a crystal size of a few nm. The ductility seems to originate from the diffusional flow of atoms along the intercrystalline interfaces.

It is well established that the plastic deformation of crystalline solids occurs by the movement of lattice dislocations and/or diffusional creep^{1,2}. Materials with immobile lattice dislocations are brittle at low temperatures. This applies to most ceramic materials (for example, Al_2O_3 and MgO) and limits their technological application. Plastic deformation of ceramic materials by diffusional creep is significant only at temperatures close to the melting point, where the rate of diffusion is high. It was the purpose of this study to make conventionally brittle ceramics become ductile by diffusional creep at low temperatures. This

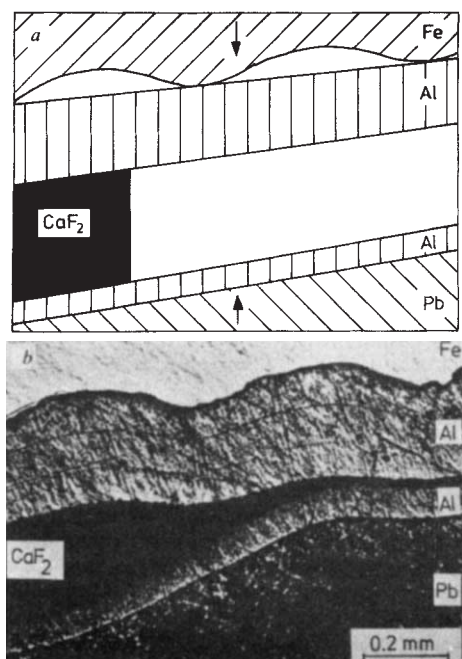


Fig. 1 *a*, Schematic drawing indicating the deformation procedure applied. The space on the right side of the CaF_2 specimen is initially empty and closes up during compression in the direction of the arrows. *b*, Plastic deformation of CaF_2 by an arrangement as shown in *a*. The deformation was done at 80 °C. The deformation time was ~1 s.

was achieved by suitably tailoring the microstructure of polycrystalline ceramics.

The deformation rate, $\dot{\epsilon}$, of diffusional creep of a polycrystalline material is given by¹⁻³

$$\dot{\epsilon} = \frac{\sigma\Omega}{d^2kT} \left(B_1 D_v + \frac{B_2 \delta D_b}{d} \right) \quad (1)$$

where σ is the tensile stress, Ω the atomic volume, d the average crystal size, B_1 and B_2 are numerical constants, D_v and D_b are the volume and boundary diffusivities, kT has the usual meaning and δ is the thickness of the boundaries. At low temperatures, where boundary diffusion predominates, one obtains

$$\dot{\epsilon} = B_2 \sigma \Omega \delta D_b / d^3 kT \quad (2)$$

According to equations (1) and (2), the diffusional creep rate of a polycrystal may be enhanced by reducing the crystal size, d , and by increasing the boundary diffusivity, D_b . This may be achieved by using nanocrystalline materials. A nanocrystalline material is a polycrystal whose crystal size is in the order of a few (1–10) nm (ref. 4). We have generated metallic materials of this type by the following two-step procedure. First, small crystallites (1–10 nm diameter) were produced by evaporating the metal in an inert-gas atmosphere. The evaporated material condensed as small crystals, which were accumulated at the surface of a cold finger held at 77 K. After restoration of high vacuum (10^{-6} Pa), the small crystals were stripped from the cold finger and funnelled into a piston and anvil device for consolidation (5 GPa, 300 K) (ref. 4). First studies⁵ of boundary diffusivity in nanocrystalline metals have suggested that the boundary diffusivity of nanocrystalline materials was about three orders of magnitude larger than short-circuit (grain boundary) diffusion in conventional polycrystals. Nanocrystalline ceramics are therefore expected to exhibit enhanced diffusional creep for two reasons: First, the reduction of the crystal size, d , from about 10 μm to ~10 nm enhances the creep rate by a factor 10^9 , as $\dot{\epsilon} \sim d^{-3}$ (equation (2)), and second, the enhanced boundary

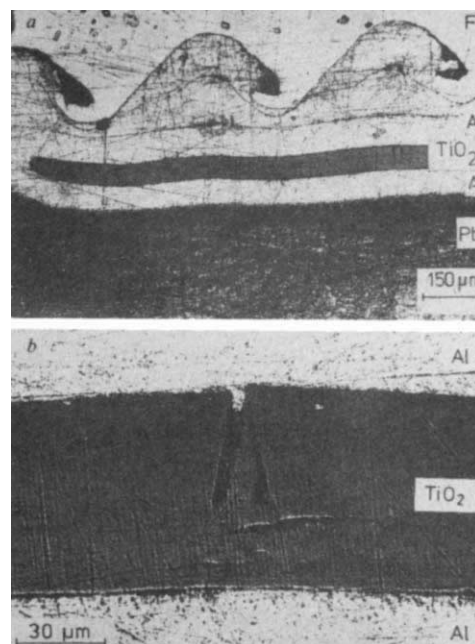


Fig. 2 *a*, Plastic deformation of TiO_2 by an arrangement as shown in Fig. 1*a*. The specimen was initially flat. The deformation time was ~1 s at 180 °C. *b*, Plastic bending of an initially flat, precracked, TiO_2 specimen (as in *a*) in the vicinity of the crack.

diffusivity may increase the creep rate by $\sim 10^3$, so that the total enhancement is $\sim 10^{12}$.

We tested the low-temperature plastic deformation of nanocrystalline ceramics by generating nanocrystalline specimens of CaF_2 and TiO_2 according to the procedure described. The nanocrystalline TiO_2 was obtained by oxidizing the small Ti crystals accumulated on the surface of the cold finger. X-ray diffraction revealed the structure to be pure rutile. No other compound of titanium and oxygen or any residual titanium could be detected. The average crystal size of all specimens was about 8 nm.

Figure 1*a* shows a cross-section through the arrangement used for the plastic deformation of nanocrystalline CaF_2 . An initially flat, square specimen was deformed by compressing it between aluminium foils supported on either side by lead or a corrugated iron piston. The plastic deformation of the nanocrystalline CaF_2 results in sinusoidal bending following the shape of the corrugated iron surface and by plastic flow to the right into a filamentary form (Fig. 1*b*).

Figure 2*a* shows the sinusoidal plastic bending of an initially flat nanocrystalline TiO_2 plate at 180 °C. The deformation procedure was performed as indicated in Fig. 1*a*.

The plastic bending of an initially flat, plate-shaped precracked specimen of TiO_2 is shown in Fig. 2*b*. The deformation results in the bending of the residual cross section and in crack

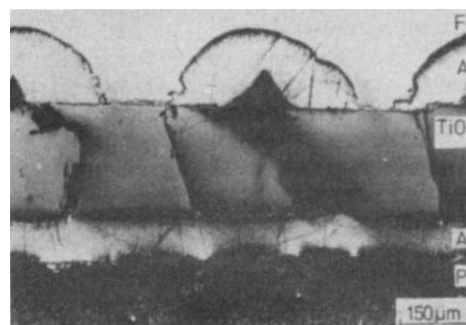


Fig. 3 Brittle fracture of a TiO_2 single crystal when deformed under the same conditions as in Fig. 2*a*.

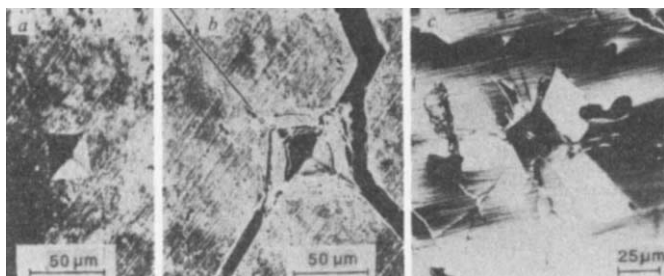


Fig. 4 *a*, Indentation of nanocrystalline TiO_2 at 20 °C (load 2 N, deformation time 10 s). The hardness of the material (deduced from the size of the indent) was 250 HV. *b*, Brittle behaviour of nanocrystalline TiO_2 (20 °C, load 2 N). The indentation was generated by reducing the loading time from 10 s (in *a*) to 1 s. *c*, Indentation of a conventional TiO_2 polycrystal under similar conditions to *a* (20 °C, load 5 N). The hardness deduced was ~800 HV. The different brightnesses of the fractured areas result from tilting relative to the incident light.

opening without crack growth. For comparison, Fig. 3 shows the deformation behaviour (brittle fracture) of a flat, plate-shaped TiO_2 single crystal under the same conditions as in Fig. 2*a*.

Figure 4*a* shows the plastic deformation of nanocrystalline TiO_2 by indenting (diamond indenter) at ambient temperature. Indentation of a conventional TiO_2 polycrystal under the same conditions as in Fig. 4*a* resulted in multiple cracking (Fig. 4*c*). If the deformation rate is larger than the diffusional creep rate, nanocrystalline ceramics should exhibit a ductile-brittle transition; Fig. 4*b* shows an example. A ductile-brittle transition was also seen if the temperature of deformation was decreased, as equation (1) predicts.

In the past, several studies of the plastic deformation of ceramics by transformational or structural superplasticity have been made. An enhanced creep of ZrO_2 and Bi_2O_3 was reported as the material underwent a structural phase transformation. Structural superplasticity⁶⁻¹¹ describes the high ductility of fine-grained ceramics such as UO_2 , MgO , Al_2O_3 , SiC and MgO Al_2O_3 . In all cases, however, the ductile deformation was limited to temperatures >1,000 °C and small strain rates (typically $\leq 10^{-4} \text{ s}^{-1}$). Hence, contrary to the reported ductility of nanocrystalline ceramics, superplastic ceramics are brittle at low temperature.

If the crystal size of a noncrystalline material is increased to a few μm or more (for example, by annealing) the properties of a conventional ceramic are retained or improved. Hence following alternative applications of the low-temperature plasticity of nanocrystalline ceramics are conceivable. Ductile ceramics may be used as a new type of ceramic material in their own right. The plasticity may also be used for a specific purpose such as processing a ceramic by extrusion or rolling. Subsequently, the material may be fully or partly converted back into a conventional ceramic. Partial conversion may be achieved by surface annealing to obtain a material exhibiting at the surface the properties (such as hardness and chemical resistivity) of a conventional ceramic. The interior of such a material would still be ductile. If grain growth were inhibited, the nanocrystalline morphology could be maintained permanently at temperatures up to the melting point.

This work was supported by the Alcoa Foundation, the Deutsche Forschungsgemeinschaft and the Bundesministerium für Forschung und Technologie. We thank Professor E. Hornbogen for valuable suggestions.

Received 16 July; accepted 2 October 1987.

- Horvath, J., Birringer, R. & Gleiter, H. *Solid St. Commun.* **62**, 319-322 (1987).
- Chung, T. E. & Davies, T. J. *Creep Fract. Engng Mater. Struct.* **295**-407 (1981).
- Crampon, J. & Escaig, B. *J. Am. Ceram. Soc.* **63**, 680-688 (1980).
- Carry, C. & Mocellin, A. *Proc. Br. Ceram. Soc.* **33**, 101-115 (1983).
- Carry, C. & Mocellin, A. in *Deformation of Ceramic Material II* (eds Tressler, R. E. & Bradt, R. C.) 391-403 (Plenum, New York, 1984).
- Panda, P. C., Raj, R. & Morgan, P. E. *J. Am. Ceram. Soc.* **68**, 522-529 (1985).
- Wakai, F., Sakaguchi, S., Kanayama, K., Kato, H. & Onishi, H. in *Ceramic Materials and Compounds for Engines* (eds Bunk, W. & Hausner, H.) 315-322 (Deutsche Keramische Gesellschaft, Bad Honnef, 1986).

Increased solubility of quartz following ferrous-ferric iron reactions

R. C. Morris & A. B. Fletcher

Division of Minerals and Geochemistry, CSIRO,
Underwood Avenue, Floreat Park, Western Australia, Australia

A knowledge of the solubility of quartz and silicates in natural waters is important to the understanding of many geological processes. Here we provide experimental data to support an earlier hypothesis¹, that during the supergene enrichment of banded iron-formations, redox reactions involving iron result in a much more rapid dissolution of quartz than would be predicted from its known solubility in water. The process is relevant not only to iron ore genesis but also to the much wider field of chemical weathering—for example, to the genesis of laterites and soils from quartz-bearing rocks. In addition, the reactions may offer a mechanism by which quartz is pseudomorphed by iron oxides.

Despite extensive experimental data²⁻⁵, there is still uncertainty about the solubility of silica species in the natural environment. Quartz, when treated to remove surface irregularities produced by crushing, has a reported solubility of 6-14 p.p.m. at 25 °C. The solubility is considered to be independent of redox conditions, and of pH in the range typical of ground water (pH 2-8). The solubility reported for amorphous silica under similar conditions is 70-150 p.p.m., an order of magnitude greater than that of quartz.

Many workers have concluded that the genesis of iron ore by supergene enrichment of banded iron-formations (BIF) is controlled, not by the mobility of iron in solution, but by the rate of removal of quartz, the slowest step in the process^{1,6-8}. Estimates for the time required to form large iron ore bodies range from a few million years⁷ to several hundred million years¹⁻⁸. The latter figure is often considered extreme, although it is not incompatible with the geological evidence of long continental exposure in Brazil⁹, the United States⁷ and Western Australia¹ during the relevant periods. Nevertheless, a shorter formation time—millions to tens of millions of years—would be easier to reconcile with the common occurrence of such iron ore deposits around the world. Support for a shorter time was found in petrographic evidence from a sample of partly enriched BIF showing unusual alteration features (ref. 1, pages 110-112, 182), which suggested that ferrous-ferric reactions might substantially increase the rate of quartz dissolution (see Fig. 1).

This hypothesis has now been tested by a series of simple dissolution experiments using finely crushed (<40 μm) quartz treated to remove surface defects^{2,3}. The crushed quartz was etched with hydrofluoric acid and alternately soaked and washed in near-boiling distilled water, over several weeks, until the dissolution rate was minimal. The powder was stored in distilled water. When samples were required, this water was decanted, fresh water was added, the mixture was shaken, and aliquots of the suspension were injected into the test solutions. These test solutions, chosen for their catalytic effects on solution rates³, contained ~2% NaCl and ~0.1% NaHCO_3 but with additional FeSO_4 , and were reacted with ~1% quartz (all wt %). Our initial tests were done at room temperature, but later experiments were at 60 °C to increase the normally slow quartz dissolution

- Nabarro, F. R. N. *Rep. Conf. Strength Solids* 75-78 (Physical Society, London, 1948).
- Herring, C. J. *J. appl. Phys.* **21**, 437-445 (1950).
- Coble, R. L. *J. appl. Phys.* **34**, 1679-1682 (1963).
- Birringer, R., Herr, U. & Gleiter, H. *Trans. Jap. Inst. Metall. Suppl.* **27**, 43-52 (1986).

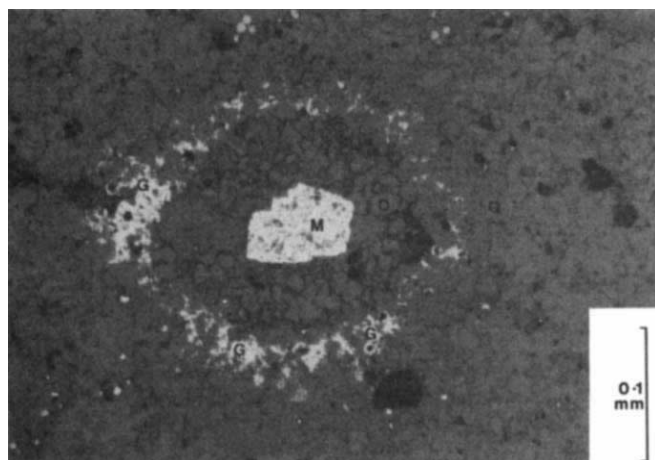


Fig. 1 Reflected-light photomicrograph showing diffuse goethite (G) forming a spherical 'halo' around an oxidized magnetite (mar-tite, M) grain, in chert from BIF. Note the increased leaching of the chert within the halo compared with that outside, which is postulated to result from ferrous-ferric iron reactions. For details see ref. 1, pages 110-112, 182.

rates. At 60 °C, the solubilities of quartz and amorphous silica are estimated² to be 25 and 250 p.p.m., respectively.

Because the reactions $\text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{e}^-$ and $\text{Fe}^{3+} + 3\text{H}_2\text{O} \rightarrow \text{Fe}(\text{OH})_3 + 3\text{H}^+$ produce acid, controlled amounts of NaOH were added to the test solutions to keep the pH in the range 2-7. Silica concentrations in solution did not increase as a result of NaOH addition. Samples in ~120-ml polyethylene bottles were placed in an oven set at 60 °C and oxidized by passing air through the solutions. Agitation of the quartz was negligible, avoiding the problem of autogenous grinding, although the bottles were shaken daily to ensure mixing. Replicate sets were allowed to react for varying times before beginning the oxidation, but with the simple apparatus used it proved difficult to keep the system anoxic beyond ~10 days. Filtered samples were diluted to 0-20 p.p.m. SiO_2 and analysed by the standard colorimetric silico-molybdate blue method; no interferences were found at these concentrations. A typical set of results is shown in Fig. 2. Further experiments involving 1, 10 and 15 wt% quartz, with the reactants continuously agitated by the slow air-intake stream (using KOH to absorb CO_2), showed similar trends but with enhanced dissolution of silica with increasing quartz surface area. In our most recent tests we added AR-grade metallic iron powder to appropriate test samples, which allowed anoxic conditions to be maintained for at least 25 days. The results from this set of tests are shown in Fig. 3.

The data for the series of tests involving immediate aeration of the iron solution are represented by a typical curve (A) in Fig. 2 and by curves 4, 12 and 13 in Fig. 3. These indicate that virtually no silica went into solution during the several weeks of the experiments, possibly because a protective layer of ferric iron was adsorbed² on the quartz grains. However, curves 7, 11 and 14 of Fig. 3, from samples containing no iron in solution, suggest that aeration (as opposed to oxidation) may have had some effect here.

Curves B and C (Fig. 2), typifying data derived from the earlier series of delayed oxidation experiments, indicate that solution of quartz is retarded during the initial non-oxidizing period (also compare curves 5 and 7 of Fig. 3), which we attribute to the increasing surface coverage by a ferrous iron/silica complex or reaction product. With oxidation, solution of silica increased rapidly, in response, we believe, to the breakdown of the iron/silica surface. The final concentration of silica in solution is clearly related to the pre-oxidation time; for example, set C (9 days) yielded higher silica concentrations than set B

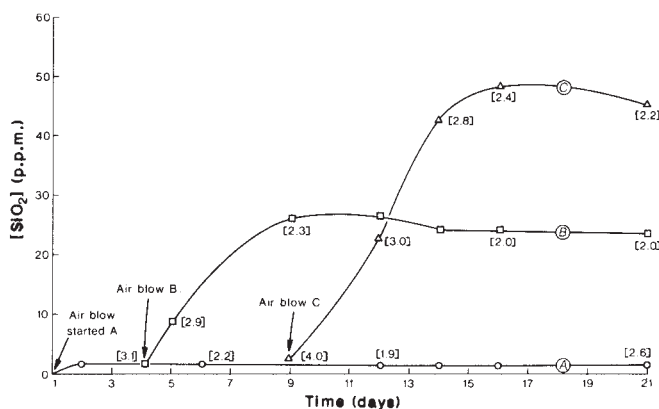


Fig. 2 Quartz solubility in identical ferrous iron solutions using ~1 wt% quartz (<40 μm), following A, aeration at the start of the experiment; B, aeration after 4 days of reaction; C, aeration after 9 days reaction. Reaction conditions: temperature, 60 °C; 1 wt% quartz; initial pH 6.6; subsequent pH values shown in square brackets.

(4 days; see Fig. 3), suggesting either increasing thickness of the reactive layer or that the ferrous complex covered a larger surface area with time.

The surface area of the quartz used for the tests in Fig. 3 was estimated from particle size data to be ~0.22 $\text{m}^2 \text{g}^{-1}$, assuming spherical particles. A value of 0.5 $\text{m}^2 \text{g}^{-1}$ is probably more realistic. The specific surface area of acid-washed quartz recovered after the Fig. 3 tests was determined as 0.32 $\text{m}^2 \text{g}^{-1}$, using the Brunauer, Emmett and Teller (krypton) technique. It is thus reasonable to assume an upper limit of 1 $\text{m}^2 \text{g}^{-1}$ for the quartz in the Fig. 3 tests. A silica concentration of 1 p.p.m. in the 110-ml test solutions is equivalent to an 0.04 nm thickness over 1 m^2 . Thus, 50 p.p.m. SiO_2 in solution is close to a 2-nm-thick layer over each grain, on average. A single molecular layer of quartz is less than 0.5 nm thick. Although these figures support the formation of a phase rather than a monolayer complex, they are critically dependent on the value of the surface area.

Identification of the surface product was attempted by subjecting coarser quartz to ferrous iron attack. These grains were washed in distilled water and acetone in a nitrogen atmosphere, and mounted for X-ray photoelectron spectroscopy (XPS). Spectra obtained indicated the presence of both iron and silicon in the surface layer of the quartz, suggesting a reaction product, but the specific material has not been identified (C. Klauber, personal communication).

In summary, our experiments tested the hypothesis that quartz/ferrous iron reactions could markedly increase quartz solubility. From more than 180 individual experiments, some repetitive, other with varying conditions and reactant levels, including oxidation by H_2O_2 instead of air, we have concluded that in ferrous iron solution an ultra-thin reaction layer or complex forms on the quartz surface limiting dissolution; this layer breaks down under oxidizing conditions, resulting in a rapid release of silica to solution, reaching values well beyond the normal solubility of quartz. Apart from trying to maintain the pH at a reasonable level, no attempt was made to simulate natural conditions. Our tests used high concentrations of ferrous iron (strong reducing conditions), followed by intensive aeration, which would not normally be expected in nature. On the other hand, in nature, lower-amplitude variation in redox conditions, with much lower ferrous iron levels, could be compensated for by the vast amount of time available. We now discuss some possible explanations for our experimental results.

First, we consider the inhibiting effect of aeration on the further dissolution of quartz in the absence of iron (curves 7, 11 and 14 of Fig. 3), noting that the level of silica in solution

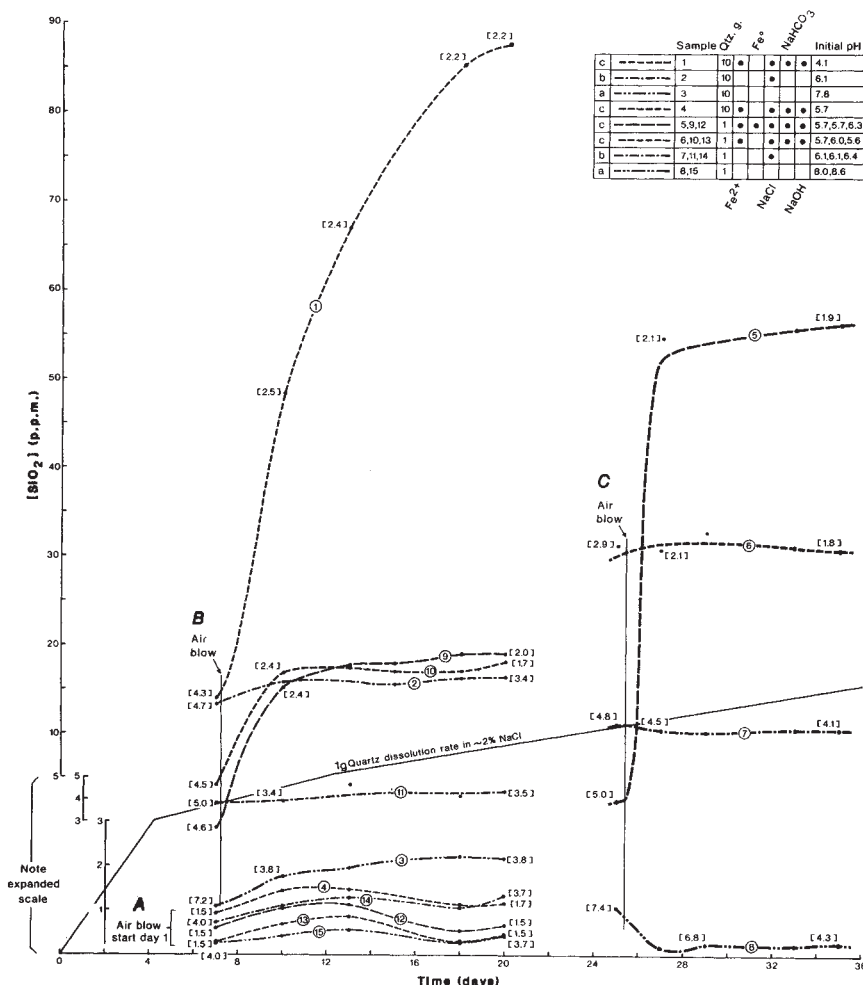


Fig. 3 A set of 15 tests run simultaneously, showing quartz solubility at 60 °C in gently agitated solutions of a, distilled water alone; b, ~2% NaCl; c, ferrous iron solutions, following A, immediate aeration, B, aeration after 7 days reaction; C, aeration after 24 days reaction. Metallic iron was added to samples 5, 9 and 12. Compare sample 6 (which oxidized slowly until day 25) with sample 5 containing metallic iron, and 10 with 9. Reaction conditions: temperature, 60 °C; pH values shown in square brackets.

at the start of the aeration remained essentially static for the remainder of the test. At least part of the decrease in pH (numbers in square brackets in Fig. 3) can be attributed to the normal slow equilibration of the surface exchange property (the higher the surface area, the more closely will the pH approach the point of zero charge of the quartz, at pH ~2; L. Warren, CSIRO, personal communication). But the apparent cessation of silica dissolution, on the other hand, is harder to explain. It may be¹⁰ that oxygen molecules (or air molecules in general) somehow displace H⁺ from the silanol (SiOH) layers, and in doing so effectively insulate the surface from further attack. If the inhibiting effect proves to be general, then the implications for weathering and other geological processes are manifold.

One possible explanation for our iron reaction experiments is that the ferrous iron formed a complex (one atomic layer thick and thus not strictly a compound) on the quartz surface, inhibiting the normal dissolution reaction. The oxidation step then converted the ferrous iron to ferric iron, which did not complex the silica, exposing 'surface defects' on the quartz, and thus promoting rapid dissolution. As the quartz surfaces were thoroughly cleaned before the tests, this explanation is unlikely, but even so, curve A (Fig. 2) and comparable tests in Fig. 3 should have shown enhanced silica dissolution if such a mechanism were valid.

A second, more likely, explanation is that, as before, a single-layer ferrous iron/silica complex formed on the quartz surface. When the pH dropped below the point of zero charge of quartz (pH ~2) as a result of the ferrous to ferric reactions listed above, a soluble ferric complex^{11,12} such as FeH₃SiO₄²⁺ was liberated, breaking down to form ferric hydroxyoxide and releasing silica to solution. This model has the drawback that Fe³⁺ is known to be strongly adsorbed² to the hydroxylated amorphous silica

surface between pH 1 and 3. Whether this holds for quartz is not known.

The original hypothesis that led to this study (ref. 1, pages 110–112, 182) suggested that a reaction between ferrous iron and quartz might form an ephemeral ferrous silicate on the grain surface which was then oxidized to form hydrous iron oxide, releasing silica to solution, and "increasing the potential solubility of quartz by a factor of ten to that of amorphous silica". As a consequence, the time envisaged for the genesis of large iron ore bodies could be decreased by as much as an order of

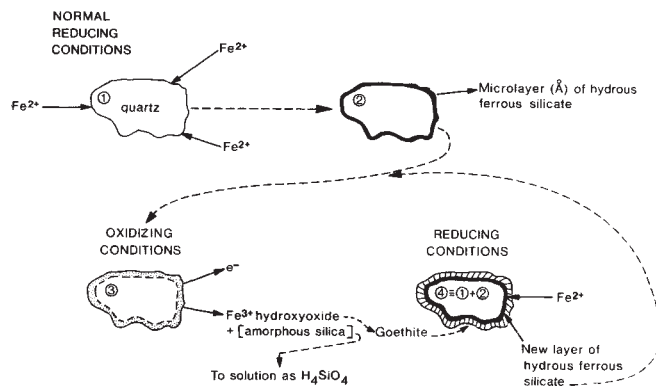


Fig. 4 The original replacement hypothesis derived from Fig. 1. Quartz (1) reacts with ferrous iron in ground water to form a tenuous hydrous ferrous iron/silica surface layer (2). Under oxidizing conditions this layer breaks down to a goethite precursor (3), releasing silica to solution at concentrations greater than possible from the dissolution of quartz alone. Repeated episodes (4) result in the eventual replacement of quartz by porous goethite.

magnitude. The enhanced solubility model, with its corollary of replacement of quartz by iron oxide, is summarized in Fig. 4.

Whether a monolayer ferrous complex or an ultra-thin but essentially gel-like compound forms is of little consequence to the hypothesis. Provided the ferrous product breaks down within the immediate vicinity of the quartz surface, as would be expected in the fine pore structure of chert, precipitation of ferric hydroxyoxide by hydrolysis should produce a coating to take the place of the dissolved silica (Fig. 1). A mechanism that could provide alternating redox conditions to continue the process in nature can be readily suggested: for example, seasonal changes from wet to dry conditions. This could produce the type of quartz replacement and strongly corroded grains typically found in laterites¹³ on granites or sandstones or in soil¹⁴. Similar effects in the replacement of BIF by supergene iron oxides¹⁵⁻¹⁷ might be promoted at depth by electrochemical processes^{17,18}, controlled by alternate wetting and drying in the outcrop. Further support for replacement comes from the tenacious iron-rich stains on quartz grains in red beds, and the yellow, brown and red coatings on the extensive sands of arid areas. Such coatings occur in both humid and arid areas throughout Australia. If the coatings are, in part, reaction effects, rather than merely grain

surface deposits, this might explain some of the frosted surfaces so characteristic of aeolian sands.

Received 26 January; accepted 9 September 1987.

1. Morris, R. C. in *Handbook of Strata-Bound and Stratiform Ore Deposits* Vol. 13 (ed. Wolf, K. H.) 73-235 (Elsevier, Amsterdam, 1985).
2. Iler, R. K. *The Chemistry of Silica* (Wiley, New York, 1979).
3. Stöber, W. in *Equilibrium Concepts in Natural Water Systems* (ed. Gould, R. F.) 161-182 (American Chemical Society, Washington, DC, 1967).
4. Siever, R. J. *Geol.* **70**, 127-150 (1962).
5. Krauskopf, K. B. *Spec. Publ. Soc. econ. Paleont. Miner.* **7**, 4-20 (1959).
6. Ruckmick, J. C. *Econ. Geol.* **58**, 218-236 (1963).
7. James, H. L., Dutton, C. E., Pettijohn, F. J. & Wier, K. L. *Prof. Pap. U.S. geol. Surv.* No. 570 (1968).
8. Trendall, A. F. *Australas. Inst. Min. Metall. Monogr.* **5**, 883-892 (1975).
9. Dorr, J. van N. II *Econ. Geol.* **59**, 1203-1240 (1964).
10. Shaw, D. J. *Introduction to Colloid and Surface Chemistry* (Butterworths, London, 1970).
11. Weber, W. J. & Stumm, W. J. *inorg. nucl. Chem.* **27**, 237-239 (1965).
12. Reardon, E. J. *Chem. Geol.* **25**, 339-345 (1979).
13. Dury, G. H. & Habermann, G. M. in *Silcrete in Australia* (ed. Langford-Smith, T.) 223-259 (University of New England, Armidale, New South Wales, 1978).
14. Abreu, M. M. & Robert, M. in *Proc. VII int. Working Meet. Soil Micromorphology* (eds Fedoroff, N., Bresson, L. M. & Courty, M. A.) 165-170 (International Society of Soil Science, Paris, 1987).
15. Morris, R. C. *Econ. Geol.* **75**, 184-209 (1980).
16. Morris, R. C. in *Banded Iron-Formation: Facts and Problems* (eds Trendall, A. F. & Morris, R. C.) 513-534 (Elsevier, Amsterdam, 1983).
17. Morris, R. C. in *Siliceous Sedimentary Rock-Hosted Ores and Petroleum* (ed. Hein, J. R.) 231-267 (Reinhold, New York, 1987).
18. Morris, R. C., Thornber, M. R. & Ewers, W. E. *Nature* **288**, 250-252 (1980).

Parallel gradualistic evolution of Ordovician trilobites

Peter R. Sheldon

Department of Geology, Trinity College, Dublin 2, Ireland

There are very few high-resolution studies of the fossil record from which to assess the relative frequency of gradualistic and punctuated evolution. Here I report some of the first detailed evidence of phyletic gradualism in benthic macroinvertebrates, based on a study of ~15,000 trilobites from central Wales. Over a period of about three million years, as many as eight lineages underwent a net increase in the number of pygidial ribs, a species-diagnostic character. The end members of most lineages have previously been assigned¹⁻³ to different species and, in one case, to different genera. In view of intermediate morphologies and temporary trend reversals, however, practical taxonomic subdivision of each lineage proved impossible. The apparent success of earlier Linnean nomenclature (with its implications of discrete species) could easily have been misinterpreted as evidence of punctuation and stasis, and it is probable that detection of many other gradualistic patterns has been hindered by ready application of binominal taxonomy to fossils.

Trilobites from the Builth inlier are among the most abundant and best known in the British Isles. Recently⁴, whilst investigating the validity of a graptolite biozone, 14,888 trilobites were obtained from seven sections in the Teretiusculus Shales (Llandeilo Series). The sections were divided into ~400 sampling localities, with a mean stratigraphic thickness of 23 cm. Given the comprehensive and rigorous taxonomic work of Hughes¹⁻³, the frequent failure to identify trilobites to specific level was wholly unexpected.

The most widespread character used to discriminate these trilobites is the number of pygidial ribs¹⁻³. Rib counts were obtained for at least one side of 3,458 specimens. Partially developed 'incipient' ribs at the posterior were recorded as a '+' and treated statistically as 0.5. The total number of ribs counted was 45,228.5. Meraspidites were excluded, and although holaspidites show a very slight positive correlation between rib count and size, changes in size are far too limited to account for the observed changes in rib count^{4,5}.

The existing species diagnoses for *Ogygiocarella* and *Cnemidopyge* (Fig. 1) proved unworkable, especially in two new

Ogygiocarella debuchii (Brongniart)

Ogygiocarella generally having 11 pygidial ribs, rarely 10 or 12.

Ogygiocarella angustissima (Salter)

Ogygiocarella with 12-14 ribs developed on pleural field, majority of individuals having 13.

Cnemidopyge parva Hughes

Cnemidopyge generally with only 5 or 6 ribs developed on pleural fields. Glabella with no median ridge.

Cnemidopyge nuda (Murchison)

Cnemidopyge with no median glabellar ridge. Pygidium generally with 7 or 8 ribs on pleural fields and up to 20 axial rings; pygidia commonly show asymmetrical development of ribs. External surface ornamented with tiny pustules.

Cnemidopyge nuda (Murchison) *granulata* Whittard

Cnemidopyge nuda generally with 8 ribs on pleural fields. External surface ornamented with small granules.

Cnemidopyge bisecta (Elles)

Cnemidopyge having median glabellar ridge, merging anteriorly with frontal glabellar spine. Pygidium with generally 8 or 9 pleural ribs commonly showing asymmetrical or irregular development of ribs. Dorsal surface of exoskeleton ornamented with small granules.

Fig. 1 Species diagnoses for *Ogygiocarella* and *Cnemidopyge* from the Builth inlier given by Hughes¹⁻³. Earlier species of each genus are given first.

stratigraphically-intermediate sections, NL and EP (Fig. 4). Rib counts for *Cnemidopyge* range from 5 to 9.5 in NL and from 5.5 to 10 in EP, thereby spanning ranges for all the species in Fig. 1. Even at individual localities the range can be considerable: for example from 5 to 9 in NL 35, and from 6.5 to 9.5 in EP 145. Mean rib counts for *Ogygiocarella* and *Cnemidopyge* often straddle the boundary between supposedly discrete successive species. Reversible shifts are common, although normally the mean remains within fairly close limits in each section. For example, for 47 localities in NL with at least three rib counts for *Cnemidopyge*, mean rib count varies only between 7.2 and 8.1. Sometimes, however, changes in mean rib count for *Cnemidopyge* encompass two 'species' in a single section. In EP, for example, mean rib count varies between 6.8 and 9.0 (65 localities, $n \geq 3$), the mean for the whole section being 8.2

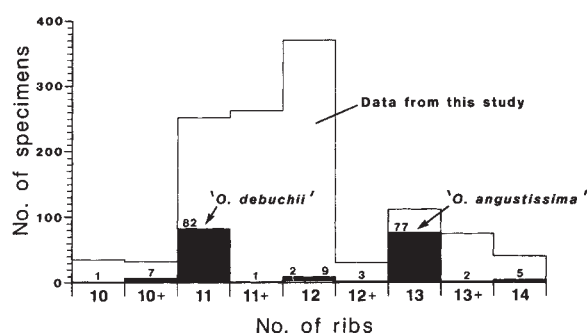


Fig. 2 Superimposed histograms of the number of ribs developed in *Ogygiocarella* comparing earlier data with results from this study. The data in black are taken from Hughes³ and relate to specimens from both the Teretiusculus Shales (left peak) and the Gracilis Shales (right peak). The peaks in this distribution have been labelled according to Hughes' taxonomic interpretation. Total number of specimens: this study, 1,211; '*O. debuchii*', 93; '*O. angustissima*', 96.

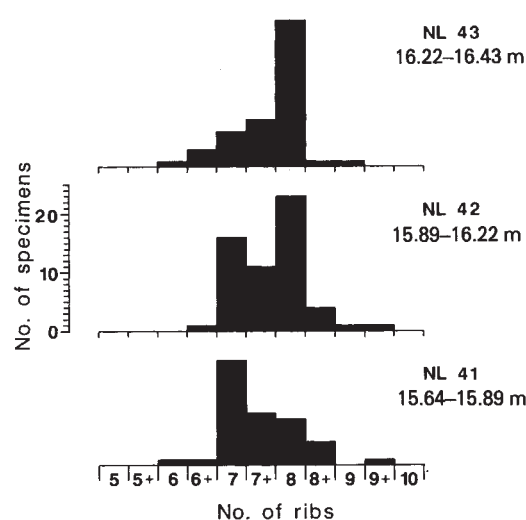


Fig. 3 Rib count distributions for *Cnemidopyge* from three successive localities in the Newmead Lane Stream Section, Teretiusculus Shales. The modes in localities NL 41 and NL 43 are clearly 7 and 8, respectively, whereas in NL 42 the distribution is somewhat bimodal, suggesting that the mode shifted from 7 to 8 within the time span represented by this assemblage. Such shifts are reversible and common. Stratigraphic position of each locality is given in meters above the base of the section. Number of specimens: 42 in NL 41, 57 in NL 42 and 45 in NL 43.

(compare with Figs 1 and 4). Figure 2 shows the data which Hughes used to justify separation of *Ogygiocarella* into two successive species. Superimposed are data from this study showing numerous 'missing links' between *O. debuchii* and *O. angustissima*, mostly from section EP.

The underlying rib count distributions of all genera are unimodal, albeit often somewhat skewed. No genus, locally at least, appears to have been represented by more than one species at any particular time. Rare bimodal character distributions are believed to reflect relatively rapid shifts in mode within the time span of the plotted assemblage (Fig. 3).

Attempts to use the median glabellar ridge as a 'presence or absence' character in diagnosis of *Cnemidopyge* species (Fig. 1) failed repeatedly. Despite assertions that *C. bisecta* arose from *C. nuda* in the Gracilis Shales¹, specimens fitting the diagnosis of *C. bisecta* were found alongside '*C. nuda*' in the Teretiusculus Shales. So many *Cnemidopyge*, however, had faint or very faint ridges that eventually an integer value for ridge development of 0 to 4 was assigned to all well-preserved glabellae ($n = 1,478$). Continuous, unimodal distributions confirmed the intraspecific

nature of this variable, whose mean altered frequently. Regression analysis showed a negative correlation between ridge development and size ($r = -0.41$, $p < 0.00001$, $n = 295$). The prominence of this ridge in many descendant adults from the Gracilis Shales was therefore probably achieved by a slight heterochronic shift involving post-displacement⁶.

Similar problems were encountered when applying existing species diagnoses to specimens of *Platycalymene*, *Nobiliasaphus* and the trinucleids *Bergamia* and *Whittardolithus*, and no new criteria emerged to permit specific separation. Nileids underwent sufficient evolution for the ends of the lineage to have

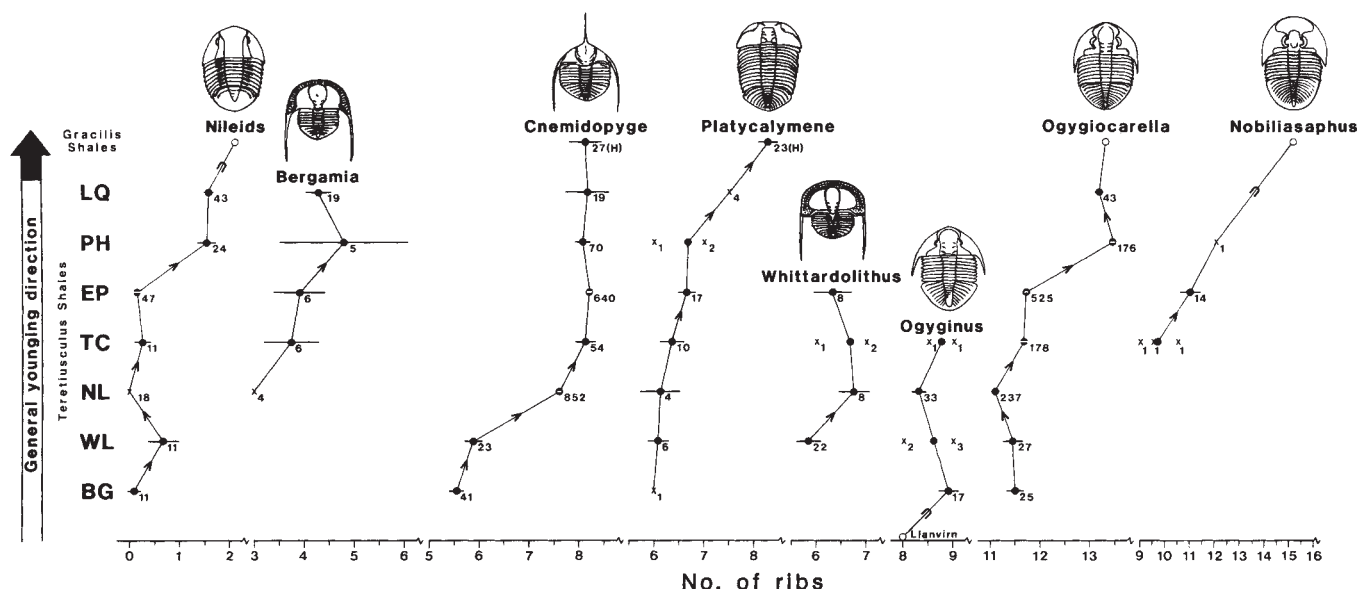


Fig. 4 Summary of changes in mean number of ribs for eight trilobite lineages in the Built inlier. Means with 95% confidence interval and number of measurements are shown by $\bullet_n$ and $\circ_n$. Approximate mean ($\circ$) based on Hughes¹⁻³ or personal observations. Data from Hughes¹ (H). Individual measurements (x_n). Successive means (x) are significantly different at the 95% confidence level. Successive means (x) are certain to be significantly different but full data are unavailable. Vertical spacing between sections is not to scale. BG: Bach-y-graig Stream Section. WL: Welfield Lodge Stream Section. NL: Newmead Lane Stream Section. TC: Trecoed Stream Section. EP: Stream Section East of Pencerrig. PH: Pencerrig House Section; LQ: Pencerrig Lake Quarry. (Full locality details in ref. 4).

been placed in different genera, *Barrandia* and *Homalopteon*³. Every character difference used to discriminate these genera and their species, however, proved, in practice, far from clear-cut⁴.

Figure 4 shows that every lineage underwent a net increase in the number of pygidial ribs. Even in *Whittardolithus* there is a net increase between WL and EP ($p < 0.05$). Significant changes, including reversals, occur at different times in different lineages. The pattern of change in *Ogygiocarella* and *Cnemidopyge* is quite different, whereas that of *Ogygiocarella* is very similar to that of the nileids.

The sections in Fig. 4 have been equally spaced because it is not possible to establish the absolute time difference between them. Field relationships and independent faunal evidence suggest that the longest stratigraphic gap is between EP and PH. Correct spacing between these sections would probably eliminate the impression of abrupt changes in the *Ogygiocarella* and nileid lineages, and future exposures between EP and PH are predicted to yield intermediate forms.

Reversals probably reflect times when some other feature, genetically uncorrelated with high rib number and unpreserved, was selected. It would be unreasonable to expect that the one feature we chose to plot was consistently the only one favoured by selection, or that it was always linked to every other favoured trait. The widespread tendency not to expect reversals, which may have been fostered by subconscious reference to Dollo's Law, is reflected in the portrayal of phyletic gradualism as unidirectional change (see for example ref. 7). Reversals complicate the theoretical arguments⁸ concerning differentiation between cases of gradualism and punctuated equilibria and, as reversals probably occur in all evolutionary lineages, many trends driven by natural selection may be indistinguishable from random walks. In this case, the fact that so many trilobite lineages show the same net trend strengthens the hypothesis that selection was involved. If change in rib number was only an ecophenotypic phenomenon one would expect far closer parallelism between lineages, as is clearly the case with changes in size⁵. At the finest resolution, increase in rib number in one genus often coincides with zero or negative change in another genus. This is to be expected as countless variables in each lineage will have been potential candidates for selection at any one time even if, in the long term, all lineages show *net* parallel evolution of a particular character.

The functional significance of pygidial ribs is unknown. Perhaps each rib indicates the presence of an underlying appendage carrying a well-developed 'gill branch'. Alternatively, or additionally, extra ribs may have conferred greater strength; several lineages show thinning of already thin exoskeletons. It is possible that rib development was pleiotropically linked to another character which was being selected, whilst variations in rib number were adaptively neutral or even slightly disadvantageous. Apparently long-sustained net trends in a single character may reflect genetic coupling to other characters having negative effects on fitness⁹.

To date, most of the better-documented cases of phyletic gradualism in invertebrates have come from pelagic microorganisms (see for example ref. 10). That gradualism should be found in benthic macroorganisms such as the Builth trilobites is perhaps not surprising, given their setting. The Teretiusculus and Gracilis Shales accumulated in persistent low-energy, dysaerobic conditions, probably in a silled marine basin several hundred meters deep⁴. The shelly benthos was almost entirely restricted to trilobites, which flourished in this stable environment for several million years. During this time a few hard-part characters, especially those maintaining high variability, eventually underwent sufficient net change for palaeontologists to credit such changes with taxonomic significance. Perhaps this kind of gradual phyletic evolution can only be sustained by organisms living in or able to track narrowly-fluctuating, slowly-changing environments, whereas stasis, almost paradoxically,

tends to prevail in more widely-fluctuating, rapidly-changing environments.

These findings represent a case of the expected, but rarely encountered dilemma facing taxonomists confronted with gradually evolving lineages and a fairly complete fossil record. To unite all the components of each lineage into single species, as a strict cladist might, would be to submerge the observed evolutionary trends under one taxon. But to subdivide a lineage into two or more arbitrary species or subspecies would give a false impression of punctuation and stasis (especially in range charts) and, in any case, could be impracticable (as here) because of temporary trend reversals. Whatever solutions to these taxonomic problems emerge, the implication from this study is that our perception of many other gradualistic patterns may have been hindered by conventional descriptive procedures, particularly the requirement to apply binominal taxonomy to fossils and the practice of lumping together specimens collected from different horizons in order to amass enough material for full 'species' description.

I thank Yolanda Barrientos, Euan Clarkson, Bill Dean, Chris Hughes and many others for discussion and encouragement, and Richard Morgan, Ian Stimpson and Diane Walker for assistance with computing.

Received 24 June; accepted 27 October 1987.

1. Hughes, C. P. *Bull. Br. Mus. Nat. Hist. (Geol.)* **18**, 39–103 (1969).
2. Hughes, C. P. *Bull. Br. Mus. Nat. Hist. (Geol.)* **20**, 115–182 (1971).
3. Hughes, C. P. *Bull. Br. Mus. Nat. Hist. (Geol.)* **32**, 109–81 (1979).
4. Sheldon, P. R. thesis, Univ. Cambridge (1987).
5. Sheldon, P. R. *Lethaia* (in the press).
6. McNamara, K. J. *J. Paleont.* **60**, 4–13 (1986).
7. Stanley, S. M. *Macroevolution, pattern and process* (Freeman, San Francisco, 1979).
8. Fortey, R. A. *Spec. Pap. Palaeont.* **33**, 17–28 (1985).
9. Charlesworth, B. *Paleobiology* **10**, 319–327 (1984).
10. Malmgren, B. A. & Kennett, J. P. *Paleobiology* **7**, 230–240 (1981).

Restoration of dysgenic muscle contraction and calcium channel function by co-culture with normal spinal cord neurons

François Rieger†, Roland Bournaud*, Takeshi Shimahara*, Luis Garcia†, Martine Pinçon-Raymond†, Georges Romey‡ & Michel Lazdunski§

* Laboratoire de Neurobiologie cellulaire et moléculaire, CNRS, 91190 Gif-sur-Yvette, France

† Groupe de Biologie et Pathologie neuromusculaires, INSERM U.153, 17 rue du Fer-à-Moulin, 75005 Paris, France

‡ Centre de Biochimie du CNRS, Parc Valrose, 06034 Nice Cedex, France

Muscular dysgenesis (*mdg*) is a spontaneous recessive lethal mutation in the mouse. The disease is characterized by a total lack of excitation-contraction coupling in embryonic skeletal muscle^{1–4}. This developmental abnormality is associated with a drastic deficiency in the expression of voltage-sensitive Ca^{2+} channels in skeletal muscle^{3–5} without alteration of the properties of voltage-sensitive Na^{+} channels⁴ or of voltage-sensitive Ca^{2+} channels in cardiac^{3,5} and neuronal cells⁵. Membrane couplings between sarcoplasmic reticulum and the transverse tubules, known as triads, were also found to be drastically altered in embryonic muscle of the homozygous mutant (*mdg/mdg*). Triads in the *mdg/mdg* muscle were less numerous, disorganized and lacked spaced densities^{3,6}. This paper shows that co-culture of *mdg/mdg* myotubes with normal spinal cord neurons re-establishes Ca^{2+} channel activity, contraction and normal triad organization. The

§ To whom correspondence should be addressed.

decrease thus cannot be due to a mutation of the Ca^{2+} channel as previously suggested⁵. Normal nerve cells may supply an essential factor to mutant muscle cells.

Figure 1a shows action potentials and contractions evoked by anodal break stimulation in control ($+/\text{mdg}$) myotubes bathed in a 140 mM Na^+ solution containing 1.8 mM Ca^{2+} . Action potentials were followed by a long-lasting after-hyperpolarization (a.h.p.) due to the activation of a Ca^{2+} -dependent K^+ conductance which is sensitive to the bee venom toxin apamin^{8,9}. Mdg/mdg myotubes also produced action potentials but without a.h.p.s and they could not contract (Fig. 1b). Figure 1c shows that after five days of co-culture of mdg/mdg myotubes with normal mouse nerve cells from the spinal cord, contractions triggered by spontaneous or evoked action potentials appeared. But only a small proportion (10–20%) of the analysed myotubes could contract. Synaptic transmission was always observed in mdg/mdg myotubes in which contraction was restored by co-culture with normal spinal cord neurons. But the induction of contractile activity in mdg/mdg myotubes did not seem to be directly linked to the function of the neuromuscular contacts as daily application of α -bungarotoxin ($0.4\text{--}20\text{ }\mu\text{g ml}^{-1}$) from the beginning of the co-culture, which does not prevent the formation of synaptic contacts but leads to a permanent block of the neuromuscular transmission¹⁰, did not affect the appearance of the contractile activity (manuscript in preparation). Figure 1d shows that slow Ca^{2+} action potentials trigger strong contractions in control ($+/\text{mdg}$) myotubes. These experiments were carried out in Na^+ -free external solution containing 2.5 mM Ca^{2+} and 140 mM tetraethyl-ammonium ion (TEA). TEA cations were used both as substitutes for Na^+ cations and as blockers of a variety of K^+ conductances.

Under the same experimental conditions, we saw no Ca^{2+} -action potential or contraction in mdg/mdg myotubes (Fig. 1e). In mdg/mdg myotubes co-cultured with normal spinal cord neurons, the observed responses to depolarizing pulses of current differed widely from cell to cell (Fig. 1f–h). They were generally of small amplitude (20–50 mV) with a slow repolarizing phase (Fig. 1h). Although these graded active responses to current were clearly different from Ca^{2+} action potentials recorded in control ($+/\text{mdg}$) myotubes, they reflected voltage-dependent Ca^{2+} channel openings as they triggered contractions, were inhibited by classical Ca^{2+} channel blockers such as Co^{2+} (data not shown) and reduced by the potent dihydropyridine derivative (+)PN 200-110 (compare Fig. 1h with Fig. 1i).

Properties of Na^+ and Ca^{2+} currents were then analysed in voltage-clamp experiments. As previously shown in myoballs⁴, the amplitude, voltage-dependence and kinetics of Na^+ currents were similar in control ($+/\text{mdg}$) and in mdg/mdg myotubes. Na^+ channel characteristics were not modified in co-cultured mdg/mdg myotubes (not shown). Clearly, properties of the voltage-dependent Na^+ channel were not affected by the dysgenic mutation, nor by the co-culture with normal spinal cord neurons.

Table 1 I_{fast} and I_{slow} characteristics in $+/\text{mdg}$?, mdg/mdg and co-cultured mdg/mdg myotubes

		$+/\text{mdg}$?	mdg/mdg	Co-cultured mdg/mdg *
Threshold (mV)	I_{fast}	-51.3 ± 7.7 (n = 10)	-50 ± 6.6 (n = 9)	-55 ± 3.8 (n = 10)
	I_{slow}	-23.8 ± 6.1 (n = 10)	absent	-22 ± 7.5 (n = 5)
$P_{\text{max}}^{\dagger}$ (mV)	I_{fast}	-31.0 ± 3.7 (n = 5)	-32.3 ± 3.5 (n = 6)	-28 ± 1.7 (n = 10)
	I_{slow}	-10.0 ± 5.9 (n = 8)	absent	-4 ± 8 (n = 5)

* Four series of co-cultures: 58 cells analysed; 26 cells with $I_{\text{fast}} + I_{\text{slow}}$ and 32 cells with only I_{fast} .

$\dagger$ Membrane potential to obtain maximum I_{Ca} .

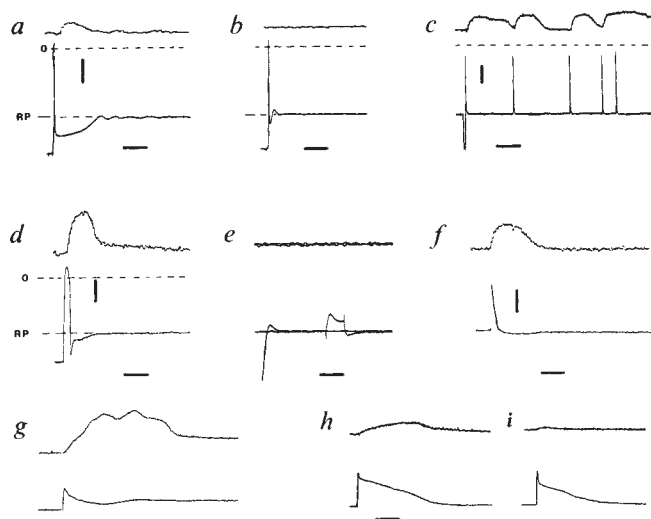


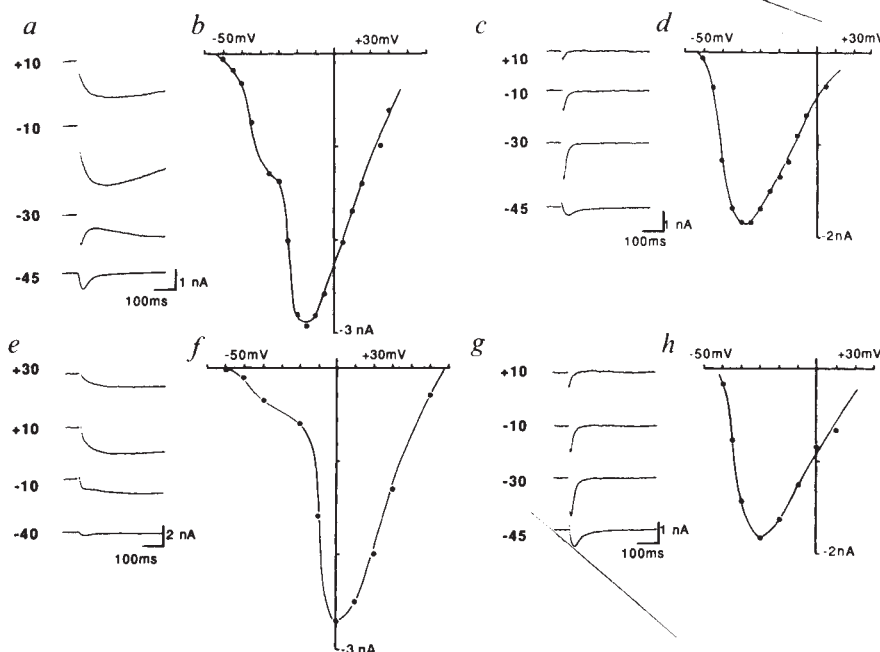
Fig. 1 Spontaneous or evoked action potentials by anodal break stimulation (lower traces) and contractions (upper traces) in arbitrary units in normal ($+/\text{mdg}$) myotubes, dysgenic (mdg/mdg) myotubes and dysgenic myotubes co-cultured with mouse spinal cord neurons. We refer to the phenotypically normal littermates of mdg/mdg new borns as $+/\text{mdg}$?, because $+/+$ and $+/\text{mdg}$ mice are indistinguishable. The myotubes were bathed either in a solution containing 140 mM Na^+ and 1.8 mM Ca^{2+} (a–c) or in a 2.5 mM Ca^{2+} , Na^+ -free solution containing 140 mM tetraethyl-ammonium ion (TEA) (d–i). a and d show $+/\text{mdg}$? myotubes; b and e, mdg/mdg myotubes; c, f–i, co-culture of mdg/mdg myotubes with mouse spinal cord neurons. In $+/\text{mdg}$? myotubes, both Na^+ (a) and Ca^{2+} (d) action potentials induced contractions and were generally followed by a long-lasting after-hyperpolarization (a.h.p.). In mdg/mdg myotubes, the a.h.p. was absent and no contractile activity was associated with the Na^+ action potential (b). Neither anodal break stimulation nor depolarizing pulses of current triggered Ca^{2+} action potential or contraction (e). c, Evoked and spontaneous electrical and mechanical activities in dysgenic myotubes co-cultured with normal spinal cord neurons. Ca^{2+} electrical responses to depolarizing stimuli and related contractions varied from cell to cell (f–h). The slow Ca^{2+} response was partially blocked and the associated contraction almost completely blocked 10 min after the external application of $0.1\text{ }\mu\text{M}$ (+)PN 200-110 (h, i). Horizontal bars, 500 ms; vertical bars, 20 mV. 0, zero voltage level; RP, resting potential level.

Methods. Primary cultures of skeletal muscle cells from 19-day-old mouse embryos $+/\text{mdg}$? or mdg/mdg were prepared as previously described² using front and hind limb muscles. Myotubes were co-cultured with nerve cells from the spinal cord under conditions in which *in vitro* innervation occurs². The spinal cord neurons from normal mouse (13–14-day-old embryos) were dissociated by mechanical trituration. Usually 5×10^6 mouse spinal cord cells per dish were added to well-differentiated myotubes. Electrophysiology and contractions: culture dishes were used immediately after the replacement of the culture medium by a specific solution. For Na^+ action potential recordings, the external solution contained: 140 mM NaCl , 1.8 mM CaCl_2 , 25 mM TEA chloride, 0.4 mM MgCl_2 , 5 mM glucose. This solution was buffered at pH 7.4 with 10 mM HEPES/KOH. Intracellular recordings were made using conventional glass microelectrodes filled with 3 M KCl, with resistances of 20–30 M Ω . The microelectrode was connected to an electrometer preamplifier (WPI M707) allowing simultaneous injection of current and recording through the same microelectrode. Contractions were recorded simultaneously with the electrical activity using the combination of a Sony TV camera and TV image analyser based on an Apple II microcomputer⁷.

Two distinct types of Ca^{2+} currents have been described in mammalian muscle cells in culture¹¹ and in a variety of other excitable cells^{12–16}. These differ in their voltage and time-dependences and in their pharmacology. The first type (I_{fast}) is characterized by a low threshold level of activation (-60 to -50 mV) and by fast activating and inactivating phases. The second type

Fig. 2 Voltage-clamp analysis of the Ca^{2+} channel activities in *a, b*, 10-day-old $+/mdg?$; *c, d*, 10-day-old mdg/mdg ; *e, f*, 14-day-old mdg/mdg myotubes co-cultured for seven days with normal mouse spinal cord neurons; *g, h*, 12-day-old mdg/mdg myotubes co-cultured for five days with mutant mouse spinal cord. In *a, c* and *e*, Ba^{2+} currents associated with step depolarizations to the indicated values from a holding potential of -80 mV. Note the presence in $+/mdg?$ myotubes of two distinct Ca^{2+} channels (*a*): a fast activating and inactivating channel with a low membrane potential threshold (-50 mV) (I_{fast}) and a slow activating channel with a higher threshold (-25 mV) (I_{slow}). *b*, Peak inward current-membrane potential relationship for the experiment shown in *a*. In mdg/mdg myotubes, only I_{fast} was present (*c*) with the same characteristics as in $+/mdg?$ myotubes. *d*, Peak I_{fast} current-membrane potential plot for the experiments shown in *c, e*. Presence of the two types of inward currents (I_{fast} and I_{slow}) in co-cultured mdg/mdg myotubes. The amplitude of I_{slow} as well as its voltage and time-dependence varied from one myotube to another. *f*, Peak current-membrane potential plot for the experiment shown in *e*. Absence of the slow Ca^{2+} conductance in co-cultured mdg/mdg myotubes with mdg spinal cord neurons. *h*, peak I_{fast} current-membrane potential plot from the experiment shown in *g*.

Methods. Ca^{2+} channel activities were measured with Ba^{2+} as charge carrier. Currents were recorded with the whole-cell variant of the patch-clamp technique¹⁷. The pipette solution contained: 100 mM CsCl, 2 mM MgCl_2 , 5 mM CaCl_2 , 6 mM EGTA. This solution was buffered at pH 7.2 with 20 HEPES/CsOH. The external solution contained: 150 mM TEA, 2.5 mM BaCl_2 . This solution was buffered at pH 7.3 with 10 HEPES/CsOH. Tetrodotoxin (1–6 μM) was systematically added to the external solution to prevent any contamination with Na^+ currents. Ba^{2+} currents were low-pass filtered at 2 kHz and sampled (5 kHz), stored and analysed on a Plessey 6220 system. Capacitive and leak components were subtracted by linear extrapolation from current observed for small depolarizing pulses.



(I_{slow}) has a higher threshold of activation (> -30 mV), a slow activating and a very slow inactivating phase. I_{slow} is the target of Ca^{2+} channel blockers of the dihydropyridine series. It is enhanced by Bay K8644 and blocked by nitrendipine, nifedipine or (+)PN 200-110.

It was previously shown that I_{fast} and I_{slow} coexist in both control ($+/mdg?$) myoballs⁴ and myotubes⁵ (Fig. 2*a, b*). Different results were previously obtained in the analysis of Ca^{2+} currents in mdg/mdg muscle cells depending on the culture treatment. Both I_{fast} and I_{slow} were missing in mdg/mdg myoballs⁴ whereas only I_{slow} was absent in mdg/mdg myotubes⁵. An explanation for this may be that the molecular structure of the fast Ca^{2+} channels in mdg/mdg muscles is altered by the colchicine treatment used to obtain rounded myoballs. Figure 2*c, d* confirms the presence of I_{fast} in mdg/mdg myotubes and the total absence of I_{slow} . Co-culture of mdg/mdg myotubes with spinal cord neurons of healthy animals re-established the slow type of Ca^{2+} current (I_{slow}) in the muscle cell. Typical Ca^{2+} currents in co-culture of mdg/mdg myotubes with spinal cord neurons are presented in Fig. 2*e, f*. The two types of Ca^{2+} currents (I_{fast} and I_{slow}) are clearly evident in Fig. 2*f*, where peak current intensities are plotted against membrane potential. There is a

strong correlation between the appearance of contraction in myotube co-cultured with normal spinal cord neurons and the presence of a slow Ca^{2+} current. Interestingly, such a restoration of a slow Ca^{2+} current is not observed when dysgenic myotubes are co-cultured with spinal cord neurons of the mutant animal (Fig. 2*g, h*).

Table 1 summarizes voltage-dependence properties of I_{fast} and I_{slow} in control $+/mdg?$, in non-innervated mdg/mdg and in mdg/mdg myotubes which have been co-cultured with normal spinal cord neurons.

A comparative ultrastructural analysis was carried out on the skeletal muscle cells in culture. Triadic junctions in control $+/mdg?$ myotubes were similar to those described in control $+/mdg?$ muscle with regularly distributed spaced densities (Fig. 3*a*). The same abnormality as in mdg/mdg embryonic muscle was found in cultured mdg/mdg myotubes, that is, an absence of clearly organized intermembranous densities in T-tubule-sarcoplasmic reticulum couplings (pseudo triads) although these densities existed as small patches in restricted contact areas (Fig. 3*b*). Interestingly, normal triadic junctions with spaced densities (Fig. 3*c*) were found to increase in number to a normal distribution (Table 2) in contracting mdg/mdg myotubes co-cultured with normal spinal cord neurons, although pseudo-triads remained scattered in the contracting, rescued myotubes (Table 2).

The results reported here indicate that dysgenic skeletal muscle cells acquire Ca^{2+} channel activity, triads, and contractile capacity after co-culture with normal spinal cord neurons. It is thus highly improbable that the gene defect in dysgenic muscle lies in the Ca^{2+} channel protein itself as previously suggested⁵ or in structural components of the triadic junction. Instead, some nerve-muscle interaction important in muscle development is defective in mdg/mdg . The most obvious possibility is that normal spinal cord neurons synthesize and secrete an essential factor that is not produced by spinal cord neurons in the mutant. Another possibility is that axonal membranes on the

Table 2 Densities of T-tubule-SR couplings for control and mutant myotubes, alone or co-cultured with normal spinal cord neurons

	Control (+/mdg?)	Mutant (mdg/mdg)	
		Normal triad	Pseudo-triad
Alone	$6.0 \times 10^{-3} \pm 1.9 \times 10^{-3}$	none	$5.3 \times 10^{-3} \pm 1.0 \times 10^{-3}$
Co-culture	$9.5 \times 10^{-3} \pm 2.1 \times 10^{-3}$	$8.0 \times 10^{-3} \pm 2.0 \times 10^{-3}$	$10 \times 10^{-3} \pm 5.8 \times 10^{-3}$

Densities are given per unit section area (μm^2) of longitudinal ultra thin sections (65 nm) of cultured myotubes.

We studied three independent muscle cultures (15 myotubes were studied in each culture).

Pseudo-triad: T tubule-SR coupling without 'periodic feet'.

Normal triad: triad with 'periodic feet'.

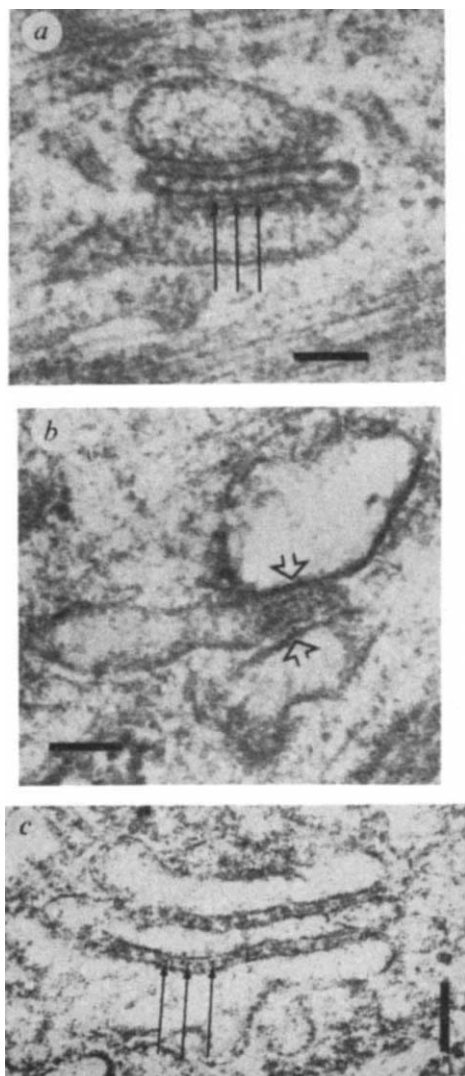


Fig. 3 *a*, Normal triadic junction in a spontaneously contracting myotube *in vitro*. Note the spaced densities (arrows) between T-tubule and sarcoplasmic reticulum ($\times 137,500$; bar, $0.1 \mu\text{m}$). *b*, A non-contracting *mdg/mdg* myotube *in vitro* showing abnormal T-tubule sarcoplasmic reticulum appositions. There are no spaced densities (wide arrows) ($\times 137,500$; bar, $0.1 \mu\text{m}$). *c*, Normal triadic junction in a contracting *mdg/mdg* myotube after co-culture with normal spinal cord neurons. Spaced densities are now visible in these mutant myotubes (arrows) ($\times 137,500$; bar, $0.1 \mu\text{m}$). Cells were fixed in the culture dishes with 2.5% glutaraldehyde, 0.5% tannic acid in a 0.1 M phosphate buffer pH 7.4 for 30 min. After 2% osmic acid post-fixation for 1 h at 4°C , and alcohol dehydration, the samples were embedded in Epon resin.

presynaptic side of normal nerve-muscle contacts produce muscle differentiation components that would be absent in dysgenic animals. But much more work is needed to identify the molecular target of the mutation as defects in the dysgenic mouse embryo are not limited to muscular ultrastructural excitability and contraction. It has also been recently observed that this pathology alters the relationship between Schwann cell and axon¹⁸.

We thank L. Houenou, Drs S. Vandaele, A. M. Tassin and R. W. Caldwell and C. Roulinat-Bettelheim. This work was partly supported by the Association des Myopathes de France, the Centre National de la Recherche Scientifique and the Institut National de la Santé et de la Recherche Médicale.

Received 1 July; accepted 20 October 1987.

1. Bowden-Essien, F. *Dev Biol.* **27**, 351-364 (1972).

2. Koenig, J., Bournaud, R., Powell, J. A. & Rieger, F. *Dev Biol.* **92**, 188-196 (1982).

3. Pinçon-Raymond, M., Rieger, F., Fosset, M. & Lazdunski, M. *Dev Biol.* **112**, 458-466 (1985).
4. Romey, G., Rieger, F., Renaud, J. F., Pinçon-Raymond, M. & Lazdunski, M. *Biochem. biophys. Res. Commun.* **136**, 935-940 (1986).
5. Beam, K. G., Knudson, C. M. & Powell, J. A. *Nature* **320**, 168-170 (1986).
6. Banker, B. M. *J. Neuropath. exp. Neurol.* **36**, 100-127 (1977).
7. Bordes, M., Bernengo, J. C. & Renaud, J. F. *Rev. scient. Instrum.* **54**, 1053-1058 (1983).
8. Hugues, M. *et al.* *EMBO J.* **1**, 1039-1042 (1982).
9. Romey, G. & Lazdunski, M. *Biochem. biophys. Res. Commun.* **118**, 669-674 (1984).
10. Cohen, M. W. *Brain Res.* **41**, 457-463 (1972).
11. Cognard, C., Lazdunski, M. & Romey, G. *Proc. natn. Acad. Sci. U.S.A.* **83**, 517-521 (1986).
12. Carbone, E. & Lux, H. D. *Nature* **310**, 501-502 (1984).
13. Nowycky, M. C., Fox, A. P. & Tsien, R. W. *Nature* **316**, 440-442 (1985).
14. Nilius, B., Hess, P., Lansman, J. B. & Tsien, R. W. *Nature* **316**, 443-446 (1985).
15. Bossu, J. L., Feltz, A. & Thomann, J. M. *Pflügers Arch. ges. Physiol.* **403**, 360-368 (1985).
16. Bean, B. P. *J. gen. Physiol.* **86**, 1-30 (1985).
17. Hamill, P., Marty, A., Neher, E., Sakman, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85-100 (1981).
18. Pinçon-Raymond, M., Murawsky, M., Mege, R. M. & Rieger, F. *Dev Biol.* (in the press).

Altered G_s and adenylate cyclase activity in human GH-secreting pituitary adenomas

L. Vallar, A. Spada* & G. Giannattasio†

CNR Center of Cytopharmacology, Department of Pharmacology and * Chair of Endocrinology, School of Medicine, University of Milan, Via Vanvitelli, 32-20129 Milan, Italy

G_s and G_i are guanine nucleotide-binding, heterotrimer proteins that regulate the activity of adenylate cyclase, and are responsible for transferring stimulatory and inhibitory hormonal signals, respectively, from cell surface receptors to the enzyme catalytic unit¹⁻³. These proteins can be directly activated by agents such as GTP and analogues, fluoride and magnesium^{2,4,5}. Decreased amounts of G_s and G_i , and even the absence of G_s , have been described⁶⁻⁹, whereas an altered G_s has been reported in a cultured cell line (UNC variant of S49 lymphoma cells¹⁰), but has never been observed in human disease states. We have found a profoundly altered G_s protein in a group of human growth hormone-secreting pituitary adenomas, characterized by high secretory activity and intracellular cyclic AMP levels. In the membranes from these tumours no stimulation of adenylate cyclase activity by growth hormone-releasing hormone, by GTP or by fluoride was observed. Indeed, the last two agents caused an inhibition, probably mediated by G_i . In contrast, adenylate cyclase stimulation by Mg^{2+} was enormously increased. This altered pattern of adenylate cyclase regulation was reproduced when a cholate extract of the tumour membranes (which contains G proteins) was reconstituted with G_s -free, cyc^- S49 cell membranes. Inasmuch as secretion from somatotrophic cells is known to be a cAMP-dependent function¹¹, the alteration of G_s could be the direct cause of the high secretory activity of the tumours in which it occurs.

Human growth hormone (GH)-secreting adenomas were studied for their secretory activities, intracellular cAMP levels and adenylate cyclase (AC) responses to regulatory agents. In some tumours (referred to as group 1) results similar to those in normal rat pituitary cells were observed, but in the cells of the remaining tumours (group 2) the results were completely different. In group 1 secretion from short-term cultured cells was $53.1 \pm 12.6 \text{ ng GH/30 min/} 2 \times 10^5 \text{ cells}$ (mean $\pm$ s.e.m.; $n=17$) under basal conditions and was stimulated 2.5-fold by growth hormone releasing hormone (GHRH) at 10^{-8} M ; cAMP levels were $2.2 \pm 0.1 \text{ pmoles/} 2 \times 10^5 \text{ cells}$ and increased 8-fold with GHRH. In contrast, in group 2 both secretion and cAMP levels were very high even under non-stimulated conditions ($246.7 \pm 78.3 \text{ ng GH/30 min/} 2 \times 10^5 \text{ cells}$ and $49.5 \pm 18.7 \text{ pmoles/} 2 \times 10^5 \text{ cells}$, respectively; $n=8$), and moreover were not appreciably increased by GHRH. The regulation pattern of AC activity was also profoundly modified in group 2

† To whom correspondence should be addressed.

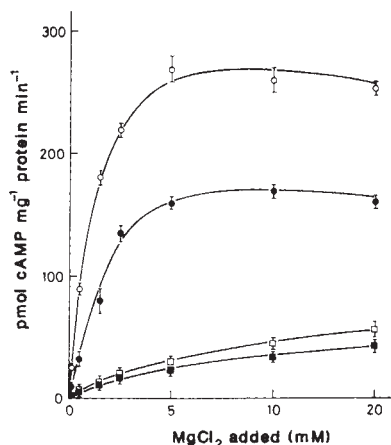


Fig. 1 Effect of MgCl_2 on adenylate cyclase activity of four human GH-secreting pituitary adenomas, two of group 1 (■—■ and □—□) and two of group 2 (●—● and ○—○). Values given are means $\pm$ s.e.m. of three determinations. Results were consistent when another six tumours of each group were tested.

Methods. Adenylate cyclase activity was determined as described¹¹ using crude membranes prepared from adenoma homogenates by centrifuging at 20,000 g for 10 min. The assay mixture contained 25 mM Tris-HCl pH 7.4, 10 mM theophylline, 1 mM cAMP, 0.2 mM EGTA, 0.15 mM $[8\text{-}^{14}\text{C}]\text{ATP}$ (40 d.p.m. pmol^{-1}), 7 mM phosphocreatine, and creatine phosphokinase (20 U ml^{-1}). The reaction was initiated by the addition of membranes (0.5 mg protein ml^{-1}) and incubated at 30 °C for 8 min. Isolation and estimation of the amount of $[8\text{-}^{14}\text{C}]\text{cAMP}$ formed were as previously described¹¹.

tumours. Thus, the stimulatory effect of Mg^{2+} was very much greater with respect to group 1 or any other cellular system reported¹²⁻¹⁴ (Fig. 1). This hyper-response to Mg^{2+} could account for the high cAMP levels observed in cultured cells because it was already appreciated at the physiological intracellular Mg^{2+} concentrations (0.5–1 mM). In contrast, the AC response to other stimulatory agents was impaired or even turned into an inhibition (Table 1). Stimulation by GHRH was less than 10% with respect to the group 1 tumour cells, and we observed a markedly decreased effect for forskolin¹⁵, a diterpene known to affect both the enzyme catalytic unit (C) and the interaction between G_s and C (ref. 16). Guanine nucleotides failed to stimulate in the 10^{-9} – 10^{-4} M range, and were inhibitory at concentrations $>10^{-7}$ M. Compare these results with those from group 1 which, in common with other cell systems^{17,18} showed stimulation by guanine nucleotides at 10^{-8} – 10^{-6} or 10^{-5} M, and inhibition only at higher concentrations. Inhibition was also evident with fluoride, another stimulatory agent¹⁹. The altered regulation of AC in group 2 tumours concerned only the stimulatory mechanism, because the response to the inhibitory hormone somatostatin (SRIF) was undistinguishable from group 1 tumour cells and normal cells¹¹ (Table 1). The amount of tissue available varied for different tumours, so restricting the number of tests possible in many cases. Results of the entire test battery are available from 12 tumours, 6 of which belonging to the group 2. In 14 tumours of the remaining 56, attribution to group 2 is supported by results in at least one test (fluoride).

The possibility that the high AC activity and the lack of stimulation by GHRH and GTP were due to small amounts of hormone and/or guanine nucleotides still bound to group 2 membranes was excluded by control experiments on extensively washed (4–5 times) membranes. Moreover, inclusion in the assay mixture of the GTP antagonist GDP β S did not reduce the enzyme activity. A similar inhibitory effect of fluoride has been found using preactivated AC (ref. 20), but this cannot explain our results because fluoride inhibition was observed even when AC activity was decreased by lowering Mg^{2+} concentration to

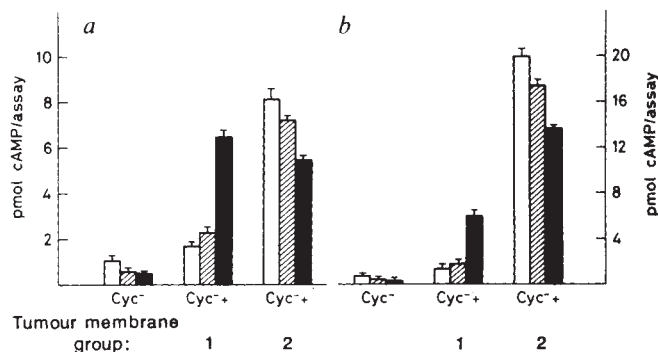


Fig. 2 Reconstitution of cyc^- S49 cell membrane adenylate cyclase activity with cholate extracts of group 1 tumour and group 2 tumour membranes. Panels *a* and *b* show two different experiments. Consistent results were obtained in two other experiments. Activities are given as pmol of cAMP formed under the experimental conditions of time, cyc^- cell membrane protein and cholate extract protein described below. Values given are the means $\pm$ s.e.m. of triplicate determinations. Open bars, 20 mM MgCl_2 ; cross-hatched bars, 20 mM MgCl_2 + 0.01 mM GTP; filled bars, 20 mM MgCl_2 + 10 mM NaF.

Methods. cyc^- S49 cells were grown in suspension culture as described²⁹. Reconstitution experiments were performed using 43,000 g particulate preparations obtained from lysed cyc^- cells²⁹. Tumour membrane 1% cholate extracts were prepared from crude membranes sedimented from tumour homogenates³⁰. Cholate extracts did not show any cAMP-forming activity and were stored at -70 °C at a protein concentration of ~ 2 mg ml^{-1} . For reconstitution, cyc^- cell membranes (43,000 g particulate preparations) and cholate extracts (350 μg of cyc^- cell membrane protein and 12.5 μl of cholate extract per 100 μl final volume) were incubated for 20 min at 32 °C in a medium containing 25 mM Na/HEPES, pH 8.0, 0.5 mM ATP, 1 mM EDTA, 2 mM MgCl_2 , 0.12% cholate was present during reconstitution. To test adenylate cyclase activity of cyc^- cell membranes, these were incubated without cholate extract under the same conditions. 20 μl of the incubated mixture was added to 80 μl of adenylate cyclase assay solution. The final mixture contained 25 mM Na/HEPES, pH 8.0, 1 mM cAMP, 0.15 mM $[8\text{-}^{14}\text{C}]\text{ATP}$ (40 d.p.m. pmol^{-1}), 7 mM phosphocreatine, creatine phosphokinase (20 U ml^{-1}), 20 mM MgCl_2 , 0.2 mM EDTA and, when required, 0.01 mM GTP or 10 mM NaF. Incubation was for 15 min at 30 °C.

as low as 0.1 mM. Therefore the group 2 tumour results are probably due to disturbance of stimulatory transmembrane signalling associated with AC, possibly involving the alteration of the stimulatory G protein. Support for this possibility was found by testing a 1% cholate extract of membranes of group 2 tumours for ability to reconstitute the cyc^- S49 cell membrane adenylate cyclase activity. The cholate extraction removes large amounts of proteins from cell membranes, including G_s (ref. 21). Owing to the lability of the catalytic unit, cholate extract has very little cAMP-forming activity, but can reconstitute MgATP -dependent adenylate cyclase activity in cyc^- S49 cell membranes, in which only the catalytic component is present²¹. The results of reconstitution experiments, performed by mixing cyc^- membranes with membrane extracts from either group 1 or group 2 tumours are shown in Fig. 2. Extracts from group 1 tumours were able to reconstitute MgATP -dependent adenylate cyclase activity stimulated by both GTP and fluoride, whereas the reconstituted activity from group 2 extracts was inhibited by either of these agents. Moreover, at the same concentration of Mg^{2+} and extract protein, the reconstituted activity was markedly higher with group 2 than with group 1 extracts. Thus the typical altered response pattern of group 2 tumours could be fully reproduced in the reconstituted system with cyc^- S49 cells. These results demonstrate that the molecular alteration of group 2 cells resides in the coupling G_s protein contained in the cholate extract.

The AC regulatory proteins G_s and G_i are known to be substrates for the bacterial toxins cholera toxin (CT) and pertussis

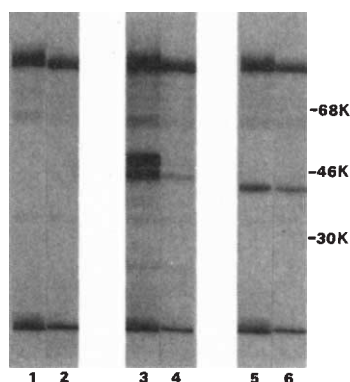


Fig. 3 SDS-polyacrylamide gel electrophoresis and autoradiography of tumour membrane proteins labelled in the presence of CT or PT and ^{32}P -NAD. Lanes 1, 3 and 5: membranes from a tumour of group 1; lanes 2, 4 and 6: membranes from a tumour of group 2. Lanes 1 and 2: membranes were incubated without toxins; lanes 3 and 4: membranes were incubated with activated CT; lanes 5 and 6: membranes were incubated with PT. The M_r markers were bovine serum albumin, 68,000; (68K); ovalbumin, 46K; carbonic anhydrase, 30K. Three other tumours of both group 1 and 2 had similar ADP-ribosylation patterns.

Methods. Tumour membranes were sedimented from the homogenates at 20,000 g for 10 min. For radiolabelling, the membranes were incubated for 10 min at 37 °C with activated³⁰ CT or PT (about 200 µg membrane proteins with 10 µg CT or 2.5 µg PT, in a final volume of 100 µl) in the presence of ^{32}P -NAD (10 µM, 24 Ci mmol⁻¹), 25 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 1 mM ATP, 0.2 mM GTP and 10 mM thymidine. Radiolabelled membranes were dissolved in the gel sample buffer containing 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl, pH 7.5, 0.02% bromophenol blue, heated for 3 min at 100 °C and applied to a 1.5 mm thick SDS-polyacrylamide slab gel (12% acrylamide) at 200 µg protein per lane and electrophoresed³¹. Gels were stained with Coomassie brilliant blue R-250, dried and autoradiographed at -20 °C using Kodak X-Omat film for 24 h.

toxin (PT) respectively, which catalyse the ADP-ribosylation of their α -subunits²²⁻²⁶. We used these toxins to better characterize the AC system in group 2 tumours. Preincubation of membranes with activated CT (which induces a marked increase of AC activity in the membrane of all other sources¹⁹, including group 1 tumours) did not result in any stimulation of enzyme activity. SDS-polyacrylamide gel electrophoresis and autoradiography of membranes incubated with the toxin and ^{32}P -labelled nicotinamide adenine dinucleotide (NAD⁺) showed that the proteins known to be ADP-ribosylated by the toxin were present only in very small amounts in membranes from group 2 tumours (Fig. 3). These proteins have relative molecular masses (M_r) of ~45,000 (45K) and 50K (ref. 21), and are presumably different forms of the G_s α -subunit²⁷. No other CT-radiolabelled proteins were visible. Therefore the stimulatory regulatory protein of group 2 tumours, unlike G_s , is not a good substrate for CT. On the other hand, in group 2 tumours, a normal G_i seems to be present. As reported²⁶, preincubation of the membranes with PT attenuated the inhibitory effect of SRIF, GTP and fluoride. Moreover, the toxin catalysed the ADP-ribosylation of a 41K protein which is probably the α -subunit of G_i (ref. 25) (Fig. 3). These results are consistent with the normal inhibitory effect of SRIF in these tumours.

In conclusion, our results demonstrate the existence of an altered G_s regulatory protein, which causes a marked change in AC activity and regulation. Inasmuch as both secretion and growth of pituitary somatotrophs (the cells that give rise to GH-secreting tumours) are known to be under the control of cAMP (refs 11, 28), a direct causal relationship between the alteration of G_s and the high secretory rate of these cells and

Table 1 Effect of different agents on the adenylate cyclase activity of human GH-secreting pituitary adenomas

Agents		Adenylate cyclase activity (pmol cAMP mg ⁻¹ protein min ⁻¹)	
		Group 1 tumours	Group 2 tumours
10 ⁻⁶ M GHRH	-	12.64 ± 1.51 (12)	102.49 ± 23.32 (8)
	+	40.60 ± 3.59	125.38 ± 29.64
10 ⁻⁵ M GTP	-	12.99 ± 2.14 (18)	137.49 ± 23.44 (11)
	+	26.30 ± 5.25	92.14 ± 14.71
10 ⁻⁵ M Gpp (NH)p	-	9.76 ± 1.73 (6)	109.84 ± 7.39 (6)
	+	21.45 ± 3.58	67.17 ± 11.08
10 ⁻² M NaF	-	12.38 ± 2.11 (48)	129.82 ± 22.58 (20)
	+	127.09 ± 16.94	94.90 ± 15.74
10 ⁻⁴ M Forskolin	-	11.35 ± 2.40 (9)	132.94 ± 18.89 (9)
	+	55.61 ± 10.68	236.53 ± 30.71
10 ⁻⁵ M SRIF	-	14.48 ± 2.82 (12)	101.97 ± 12.47 (12)
	+	10.53 ± 2.22	68.96 ± 7.52

Values given are the means ± s.e.m. of adenylate cyclase activity observed in different tumours, in the presence or absence of the agent. Numbers in parentheses indicate the number of tumours tested for each agent. Adenylate cyclase activity was determined on crude membrane preparations as described in legend to Fig. 1. The concentration of MgCl₂ added to the assay mixture was 1.5 mM. In the experiment testing the effect of GHRH and SRIF, 0.01 mM GTP was used in the assay.

their growth is possible. To our knowledge this is the first case of a disease state in which an altered G protein is expressed.

We thank Dr M. Giovanelli for supplying human adenomas, Dr G. D. Aurbach for the gift of *cyc*⁻ S49 cells, Dr J. Meldolesi for critical revision of the manuscript, and G. Gatti and C. Muca for technical support. This work was partially supported by a grant from the Italian National Research Council, Special Project 'Oncology'.

Received 16 July; accepted 15 October 1987.

- Rodbell, M. *Nature* **284**, 17-22 (1980).
- Gilman, A. G. *Cell* **36**, 577-579 (1984).
- Gilman, A. G. *Trends Neurosci.* **9**, 460-463 (1986).
- Codina, J. et al. in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* Vol. 17 (eds Greengard, P., Robison, G. A., Paoletti, R. & Nicolsia, S.) 111-125 (Raven, New York, 1986).
- Codina, J., Hildebrandt, J. D., Birnbaumer, L. & Sekura, R. D. *J. biol. Chem.* **259**, 11408-11418 (1984).
- Levine, M. A., Eil, C., Downs Jr, R. W. & Spiegel, A. M. *J. clin. Invest.* **72**, 316-324 (1983).
- Hildebrandt, J. D. et al. *Nature* **302**, 706-709 (1983).
- Jakobs, K. H. & Schultz, G. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3899-3902 (1983).
- Gawler, D., Milligan, G., Spiegel, A. M., Unson, C. G. & Houslay, M. D. *Nature* **327**, 229-232 (1987).
- Schleifer, L. S., Garrison, J. C., Sternweis, P. C., Northup, J. K. & Gilman, A. G. *J. biol. Chem.* **255**, 2641-2644 (1980).
- Spada, A., Vallar, L. & Giannattasio, G. *Endocrinology* **115**, 1203-1209 (1984).
- Rude, R. K., Baker, A. J. & Watts, R. B. *Endocrinology* **113**, 1348-1355 (1983).
- Oldham, S. B., Rude, R. K., Molloy, C. T., Lipson, L. G. & Boggs, T. T. *Endocrinology* **115**, 1883-1890 (1984).
- Bockaert, J., Cantau, B. & Sebben Perez, M. *Molec. Pharmacol.* **26**, 180-186 (1984).
- Seamon, K. B., Padgett, W. & Daly, J. W. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3367-3367 (1981).
- Yamashita, A., Kurokawa, T., Higashi, K., Danura, T. & Ishibashi, S. *Biochem. biophys. Res. Commun.* **137**, 190-194 (1986).
- Yamamura, H., Lad, P. M. & Rodbell, M. *J. biol. Chem.* **252**, 7964-7966 (1977).
- Steer, M. L. & Wood, A. J. *J. biol. Chem.* **254**, 10791-10797 (1979).
- Spiegel, A. M. & Downs, R. W. *Jr Endocr. Rev.* **2**, 275-305 (1981).
- Manganiello, V. C. & Vaughan, M. *J. biol. Chem.* **251**, 6205-6209 (1976).
- Northup, J. K. et al. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6516-6520 (1980).
- Moss, J. & Vaughan, M. *A. Rev. Biochem.* **48**, 581-600 (1979).
- Katada, T. & Ui, M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3129-3133 (1982).
- Kahn, R. A., Katada, T., Bokoch, G. M., Northup, J. K. & Gilman, A. G. in *Post-Translational Covalent Modifications of Proteins* (ed. Johnson, B. C.) 373-395 (Academic, New York, 1983).
- Katada, T., Bokoch, G. M., Northup, J. K., Ui, M. & Gilman, A. G. *J. biol. Chem.* **259**, 3568-3577 (1984).
- Ui, M. *Trends Pharmacol. Sci.* **5**, 277-279 (1984).
- Robishaw, J. D., Smigel, M. D. & Gilman, A. G. *J. biol. Chem.* **261**, 9587-9590 (1986).
- Billestrup, N., Swanson, L. W. & Vale, W. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6854-6857 (1986).
- Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L. & Gilman, A. G. *J. biol. Chem.* **252**, 5761-5775 (1977).
- Rich, A. K. et al. *J. biol. Chem.* **259**, 7893-7901 (1984).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).

Identification and sequence of a fourth human T cell antigen receptor chain

Elwyn Y. Loh^{*†}, Lewis L. Lanier[§], Christoph W. Turck^{||},
Dan R. Littman[¶], Mark M. Davis^{*‡}, Yueh-Hsiu Chien^{*}
& Arthur Weiss^{||¶}

^{*} Department of Medical Microbiology, [†] Department of Medicine, and [‡] The Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California 94305, USA

[§] Becton Dickinson Monoclonal Center, Inc., Mountain View, California 94043, USA

^{||} Department of Medicine, and [¶] Department of Microbiology and Immunology, Howard Hughes Medical Institute, University of California, San Francisco, California 94143, USA

Thymus-derived lymphocytes (T cells) use clonally distributed antigen receptors to recognize peptide fragments associated with products of the major histocompatibility complex (MHC) (refs 1–4). On most murine and human T cells the T cell receptor (TCR) is composed of disulphide-linked α and β chains ($TCR_{\alpha/\beta}$), each of which contains constant and variable domains, and which are associated with the invariant chains of the CD3 complex^{5,6}. It has been demonstrated, however, that a distinct CD3-associated TCR is expressed on a small subset of T cells or immature thymocytes which fail to express either CD4 or CD8 (refs 7–14), the molecules

associated with class II or class I MHC antigen recognition. Instead of $TCR_{\alpha/\beta}$, these cells express heterodimers of γ and δ chains ($TCR_{\gamma/\delta}$). The genes encoding α , β , and γ have been isolated and characterized^{15–17}. A new murine T cell receptor (C_x) gene which undergoes rearrangement and expression early during T cell ontogeny has recently been identified 5' of the murine $J_{\alpha}C_{\alpha}$ gene locus¹⁸. Here we isolate and sequence the homologous transcript from PEER, a human cell line that expresses a $TCR_{\gamma/\delta}$, and show that it encodes a protein with characteristic V, D, J, and C segments. Using probes derived from this transcript, we have shown that both PEER and MOLT-13, another $TCR_{\gamma/\delta}$ -expressing cell line, rearrange this locus and express two sizes of transcripts differing in the 3' untranslated region. Using a synthetic peptide derived from the deduced C region sequence, we have prepared antisera that precipitates the δ chain of the TCR from both PEER and MOLT-13, thus demonstrating that C_x and its human homologue code for the δ chain of the TCR.

To determine whether human cells which express $TCR_{\gamma/\delta}$ products transcribe the human homologue of the murine $J_{\alpha}C_{\alpha}$ locus, northern blots containing PEER RNA were hybridized with a murine C_x probe and two sizes of transcripts were found (data not shown). To characterize these transcripts, we screened a size-selected λ gt10 complementary DNA library constructed from PEER messenger RNA¹⁹ with the murine C_x probe. Plaques hybridizing with this probe were detected at a frequency of 1 in 10,000. Ten positive phages were characterized by partial restriction digests. Several size classes of cDNA were found. One set of three phages had two *Eco*RI fragments, ~500 and

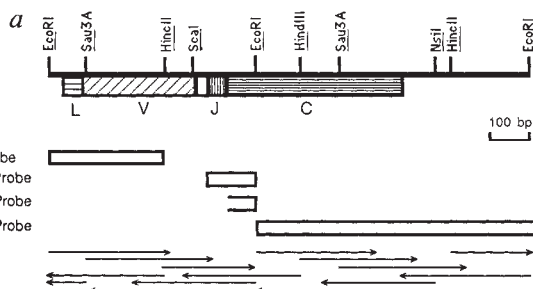
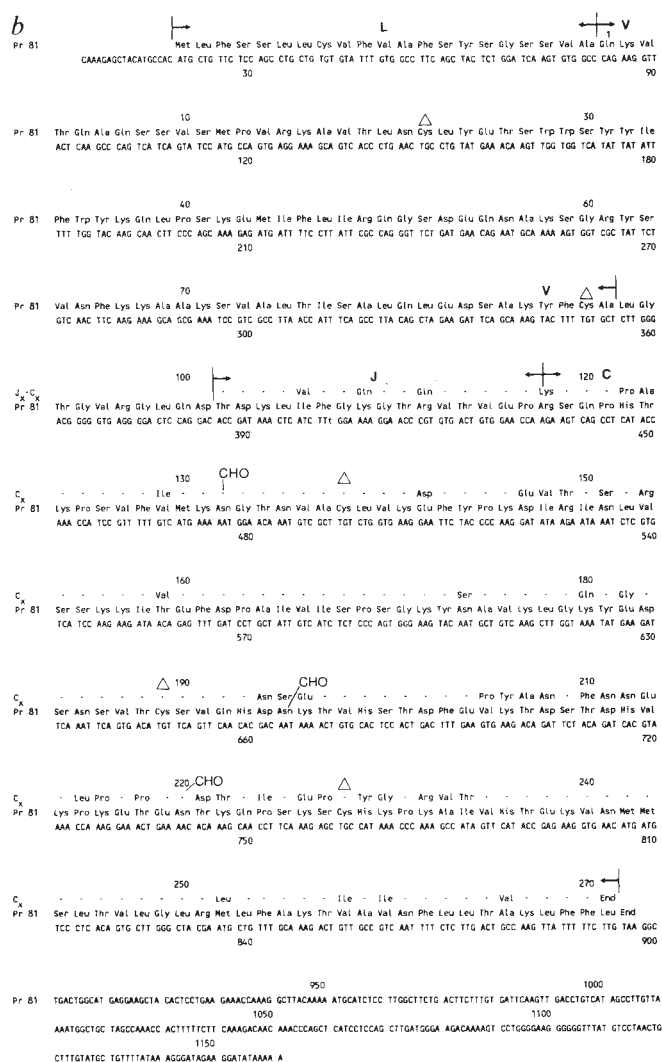


Fig. 1 Partial restriction map, probe fragments, sequencing strategy, and sequence of Pr 81. **a**, A schematic drawing and **b**, the nucleotide and predicted amino-acid sequences of the Pr 81 fragment isolated from the *Eco*RI site in λ gt10. The sequences are compared to the murine C_x sequence¹⁸. Separation into L, V, J, and C regions is by similarity to V_{α} sequences and murine C_x sequences¹⁸. The region between V and J is likely to contain D region sequences as described in the text. The V probe is an *Eco*RI–*Hinc*II fragment from Pr 81. The J–C probe extends from cDNA positions 376–510 in the sequence shown in **b** and is actually from another cDNA clone. The 5' C probe is from an incompletely processed cDNA and is a 355-bp *Eco*RI fragment that includes cDNA positions 434–510 from **b**, but also includes dissimilar 5' sequences from the intron as indicated by the unclosed box. The 3' C probe is from a cDNA isolate with an *Eco*RI fragment that includes the region shown, but also extends an additional 700 bp beyond the poly(A) tail of Pr 81. Fragments of DNA were subcloned into either Bluescript (Stratagene) or pGem3 (ref. 20) (Promega Biotec) vectors and sequenced as double-stranded DNA using either Klenow²¹ or the modified T7 polymerase²² (US Biochemical). The arrows show the collection of sequencing runs from fragments of Pr 81. The amino acids in **b** are from the longest open-reading frame and start at the ATG at nucleotide position 19. The numbering of the protein sequence is from the probable processing point at the N terminus. Cysteine residues are indicated by a triangle. Possible N-glycosylation sites are indicated by 'CHO'. cDNA position 402 has been sequenced for three different cDNAs: Pr 81 has a C, but the other two isolates have an A at that position. This is likely to be a reverse transcriptase error^{23,24} in the cloning of Pr 81 as we suggest that the other allele has been deleted in PEER (see discussion of the Southern data). The substitution does not affect the protein sequence.



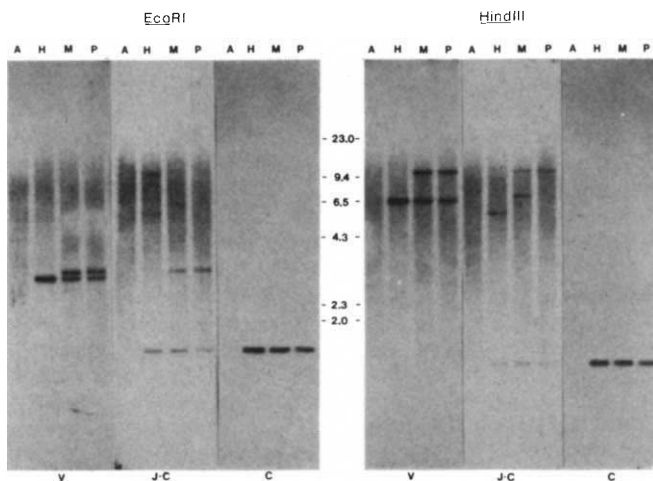


Fig. 2 Southern blot analysis of T-cell leukaemia cell lines. Lanes show DNA (10 mg) digested with *EcoRI* and *HindIII* from A, HPB-ALL, H, HL60, M, MOLT 13; P, PEER. Positions of *HindIII*-cut lambda markers are shown in kb. Probes are described in Fig. 1a, with C indicating a 5' C_δ probe. Hybridization was in 5X SSPE (NaCl, 0.75 M; NaH_2PO_4 , 0.05 M; Na_2EDTA , 0.005 M) 50% formamide, 4X Denhardt's solution, 100 $\mu\text{g ml}^{-1}$ salmon sperm DNA and 10% dextran sulphate. Filters were washed with 0.2X SSPE at room temperature for V and J_δ - C_δ and at 55 °C for C_δ probes.

700 base pairs (bp) in length. The restriction map of Pr81, which had the longest 5' end, is shown in Fig. 1a and its sequence in Fig. 1b. The sequence of Pr 81 has an open-reading frame beginning with a methionine at nucleotide position 19 and which codes for a protein of 292 amino acids. The unmodified relative molecular mass (M_r) of the peptide chain would be 31,000 (31K). From its homology with human V_α and the murine C_x protein^{17,18}, the protein can tentatively be divided into leader, V, D, J, and C regions. There are two potential N-glycosylation sites at amino acid positions 132 and 195 in the C region. Like the other TCR gene¹⁵⁻¹⁷, Pr 81 has a cysteine residue for inter-chain disulphide bonding and a conserved lysine in the putative transmembrane region. For simplicity, we will refer to both C_x and its human equivalent as the δ chain. The evidence for this will be discussed.

When the V region of Pr 81 is compared to other human V regions, most amino acids that are highly conserved in V_α (ref. 17) are present in Pr 81. The most similar human V-region reported to date is from HPB-MLT (ref. 25), which shares 75% nucleotide and 59% amino acid similarity with Pr 81, a degree of difference comparable to that between V_α families. The genomic organization of the murine C_δ - C_α' locus, with the J_δ and C_δ located 5' of $J_\alpha C_\alpha$, suggests that the δ chain could potentially use the same V_α regions as the α chain¹⁸.

There is a stretch of ten amino acids between the end of the V region and the beginning of the J region that probably contains a D region by analogy with the murine sequence¹⁵. The nucleotide sequence of this region is notable for a G-rich segment which is very similar to both human D_β 2.1 (12 out of 16 bp identical) and human D_β 1.1 (9 out of 12 bp identical) germline sequence¹⁷. Surprisingly, this sequence is not very similar to the murine germline D_δ region (Chien *et al.*, in preparation).

As shown in Fig. 1b, the mouse and human C regions are very similar; several features shared by murine C_δ and C_α (ref. 18) are also found in the human sequences. For example, both Pr 81 and C_α have short cytoplasmic hydrophobic carboxy-termini compared with the longer and more polar tails of C_β and C_γ (refs 15-17). The distribution of the similar amino-acid sequences in the C regions suggests an evolutionary and possible functional relation between C_α and C_δ , and between C_β and

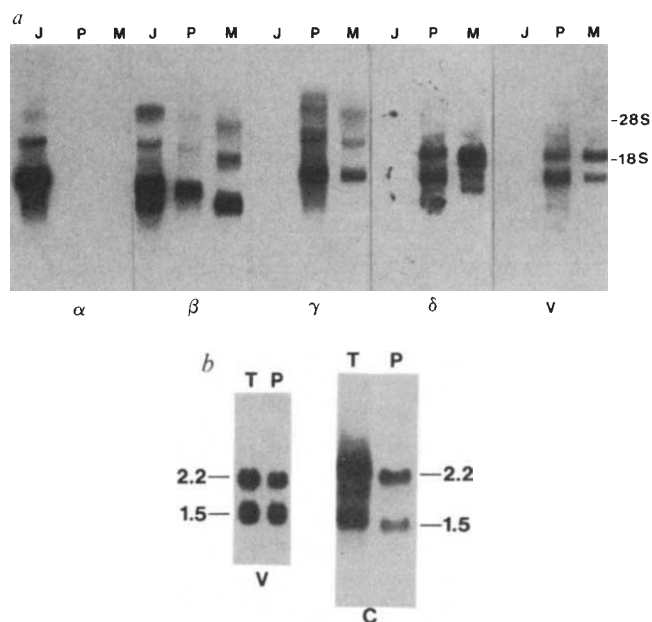


Fig. 3 Northern blot-hybridization analysis of three T-cell leukaemia cell lines. Total RNA (15 μg) was separated on 1% agarose gels, transferred and hybridized using probes for full length α , β and γ -chains^{9,19}. The 3' C_δ region probe and V_δ region probe are described in Fig. 1a. The positions of the 18S and 28S RNA are indicated. a, RNA from J, Jurkat; P, PEER; M, MOLT-13 cells. b, RNA from P, PEER and T, thymic TCR $_{\gamma/\delta}$ -bearing cultured cells. Thymic TCR $_{\gamma/\delta}$ -bearing cells were isolated and cultured in IL-2 (ref. 28).

C_γ (ref. 18), which could be important in the physical pairing of α to β and δ to γ .

The pattern of rearrangement of the δ -chain locus in TCR $_{\gamma/\delta}$ -bearing cells was compared to a TCR $_{\alpha/\beta}$ -bearing cell line. Figure 2 shows a Southern blot of DNA extracted from four cell lines and cut with *EcoRI* or *HindIII* and hybridized with V_δ , J_δ - C_δ , or C_δ region probes from Pr 81. HPB-ALL, a T-cell leukaemia line expressing TCR $_{\alpha/\beta}$ on the cell surface²⁶, has deleted the homologous sequences which are detected by all three probes deleted, as also observed in the similar region in the murine gene^{18,27}. The V region probe identifies a single band in HL60 (germline configuration), and both PEER and MOLT-13 have an additional rearranged band. The C_δ region probe includes sequences 5' to the internal *EcoRI* and *HindIII* sites and hybridizes to only one band for all three cell lines, suggesting C_δ is present as a single copy. The C_δ region probe cannot recognize rearrangements because it is far enough 3' to the J_δ region for the intervening sequence to contain both *EcoRI* and *HindIII* sites. The J_δ - C_δ region probe hybridizes to all the bands identified by the C_δ probe, as expected. In HL60, it identifies two additional bands when the DNA is cut with *EcoRI* and one band when cut with *HindIII*. The extra band with *EcoRI* is due to a rearrangement in the myelomocytic cell line, because other non T-cell cell lines have only the 6.1 kilobase (kb) band (unpublished result). MOLT 13 has two bands due to J_δ -region hybridization, one of which also hybridizes to the V_δ probe, as expected for the fragment containing the V - D_δ - J_δ region. The additional J_δ hybridizing band is unlike either band in HL60 and probably represents a recombinational event on the other chromosome. Unlike MOLT 13, PEER has one J_δ -hybridizing band identical to the V band. The absence of a second J_δ hybridizing band in PEER could result from the deletion of part of the second allele. This is supported by the decreased intensity of the PEER C_δ region bands which suggests that this deletion also includes the C_δ region.

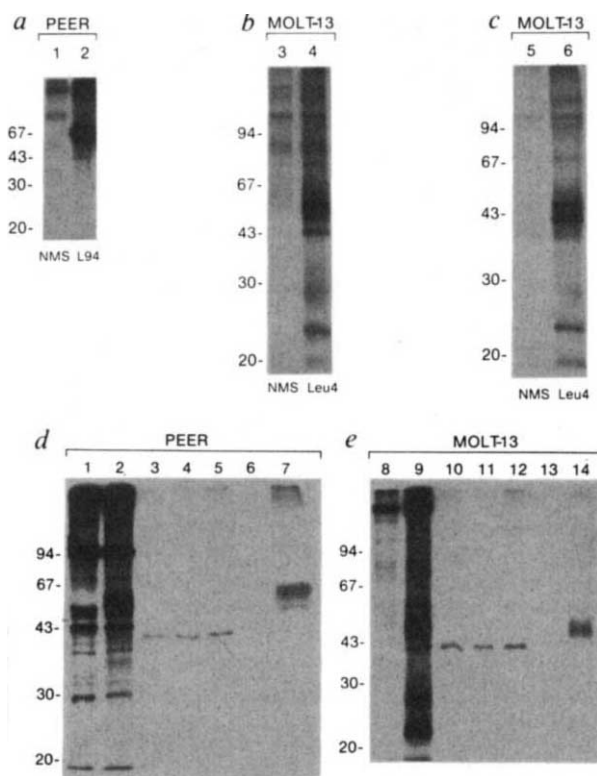


Fig. 4 Immunoprecipitates of the TCR γ/δ from the leukaemic T cell lines PEER and MOLT-13. *a*, PEER cell surface radio-iodinated proteins were solubilized in 1% NP40 and immunoprecipitated with either normal mouse serum (NMS) or mAb L94 ascitic fluid-coated formalin-fixed staphylococcus aureus (staph A) as described^{19,30}. Under these conditions, the association between CD3 and TCR chains is disrupted. *b, c*, Radio-iodinated cell surface proteins derived from the cell line MOLT-13 were solubilized in 5 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate). Immunoprecipitates were prepared with NMS- or anti-CD3 (Leu 4)-coated staph-A. In 5 mM CHAPS, the association between CD3 and TCR chains is preserved⁹. Immunoprecipitates were analysed under non-reducing (*b*) and reducing (*a* and *c*) conditions. *d, e*, Immunoprecipitates containing TCR γ/δ chains were prepared from radio-iodinated PEER (*d*) or MOLT-13 (*e*) cell-surface proteins as above. L94 immunoprecipitates from PEER and anti-CD3 immunoprecipitates from MOLT-13 were isolated under reducing and non-reducing conditions, respectively. A sample of each immunoprecipitate was denatured in 1% SDS at 56 °C for 30 min, diluted with 5 vols of 1.5% NP40 and an aliquot re-immunoprecipitated with staph-A coated with each of three rabbit antisera raised against a peptide synthesised from the predicted C γ sequence or with a previously described rabbit anti-C γ antiserum¹⁴. Immunoprecipitates were analysed by autoradiography following SDS-PAGE on 10% gels. Lanes (1, 8) NMS; (2) L94; (9) anti-CD3; (3-5, 10-12) rabbit anti-C δ peptide; (6, 13) normal rabbit serum; (7, 14) rabbit anti-C γ peptide. The relative mobilities of standards are shown in the margins.

Methods. The C δ peptide, residues 159-187, was synthesized on an Applied Biosystems Model 430A peptide synthesizer and cleaved from the resin and deprotected using liquid nitrogen fluoride. Purification of the peptide by preparative HPLC was carried out with a V γ dac C18 column (1×25 cm) using a gradient of 5-65% acetonitrile in water, 0.1% trifluoro-acetic acid. The peptide identity was confirmed by gas phase sequencing.

The expression of TCR transcripts in the TCR γ/δ -bearing leukaemic cells PEER and MOLT13 was compared to the TCR α/β -bearing leukaemic cell line Jurkat. Northern blot analysis shows that PEER and MOLT13 do not express α -chain transcripts (Fig. 3a). Whereas PEER expresses a full-length 1.3 kb mRNA for β -chain, MOLT13 has only a 1.0 kb B-chain transcript, presumably the result of a D-J rearrangement. Both PEER and MOLT-13 contain abundant levels of γ chain mRNA. High concentrations of α - and β -chain transcripts are detectable in Jurkat, but γ chain mRNA is detected only in small amounts. A C δ region probe from Pr 81 hybridizes to two bands at 2.2 and 1.5 kb on a northern blot of RNA from PEER (Fig. 3a). Both bands hybridize to a V δ region probe. Two points suggest that a possible explanation for the two transcripts is the existence of two poly(A) addition sites. First, we have found a class of cDNAs that does not end with poly(A) at position 1,156 like Pr 81, but has a poly(A) tail ~700 bp further 3' (data not shown). Second, a probe from the 3' end of this longer cDNA hybridizes only to the larger transcript (data not shown).

Hybridization of a C δ probe to a northern blot of RNA from MOLT-13 gives a more complex pattern with four bands at 2.2, 1.9, 1.5 and 1.3 kb (Fig. 3a). The bands at 2.2 and 1.5 are the same size as those of PEER, and presumably also result from the variable use of different poly(A) addition sites. The two other bands do not hybridize to a Pr 81-derived V region probe and therefore have a different or truncated 5'-end. One possibility, by analogy to the β locus, is that the 1.3 and 1.9 kb transcripts originate from a D-J rearrangement on the other allele deleted from PEER but not MOLT-13. The band intensity for hybridization to the Pr 81 V region differs in PEER and MOLT-13, suggesting there is different regulation of a preferred poly(A) site, as found in the secreted versus membrane forms of C μ (ref. 29).

It is remarkable that distinct cells expressing TCR γ/δ apparently express the same V region. This can be seen in Fig. 3a, where a V-region probe from Pr 81 identifies transcripts of identical size in PEER and MOLT-13 of 2.2 and 1.5 kb. Furthermore, a V region probe from Pr 81, hybridized to RNA from

polyclonal TCR γ/δ -bearing thymic cells expanded in IL-2 (ref. 28), also recognizes a significant number of transcripts (Fig. 3b). The Pr 81 V region is therefore expressed in a substantial fraction of TCR γ/δ -bearing thymocytes. Preferential use of V genes in the expression of δ chains could result from functional constraints (such as those needed for association with V γ , or recognition of particular antigens) or from genetic constraints (such as a proximity to C δ , limited numbers of V regions, or particular recognition sequences).

Immunoprecipitation was used to demonstrate that we had identified a cDNA encoding the human TCR δ chain. The human T cell line PEER expresses a CD3-associated TCR γ chain of 55 K variably observed to be associated with a δ partner chain^{9,12,19}. A monoclonal antibody (MAB) L94 which reacts with clonotypic determinants of the TCR γ/δ -bearing receptor on PEER (ref. 30) and gives a more reliable isolation method of γ and δ chains from PEER. Immunoprecipitates prepared with L94 from 1% NP40 lysates of PEER contain the 55K γ chain and a 41K δ chain, but not the associated CD3 proteins (Fig. 4a).

The cell line MOLT-13 expresses CD3 but does not bind MAB WT31, a MAB which reacts with non-polymorphic determinants of TCR α/β (ref. 31). CD3 immunoprecipitates prepared from MOLT-13 under conditions which preserve CD3/TCR association contain, in addition to CD3 chains, a diffuse 45K band representing a γ chain product and a non-disulphide-linked 41K band similar to the δ chain on PEER (Figs. 4b, c and e). The mobility of the putative δ chain of MOLT-13 changes on reduction of sulphhydryl bonds, suggesting the presence of intrachain disulphide linkages, as previously observed in the δ chain on other cells¹².

To determine whether the 41K δ chains on PEER and MOLT-13 were encoded by the gene identified by PR 81, rabbit antisera were raised against a synthetic peptide derived from the C region sequence predicted by PR 81, residues 159-187. Immunoprecipitates were prepared from PEER lysates with the clonotypic L94 MAB, denatured by SDS and heating, and re-immunoprecipitated with antisera from three rabbits immunized with the C δ

peptide. Antisera from all three rabbits reacted with the 41K band from the L94 immunoprecipitate (Fig. 4d). For comparison, a rabbit antiserum reactive with a C_γ region peptide sequence was used. This antiserum reacted with the 55K γ chain product contained in the L94 immunoprecipitate.

As a clonotypic MAb against the MOLT-13 TCR was not available, we performed the same analysis with a CD3 immunoprecipitate of cell-surface proteins derived from MOLT-13 under conditions preserving the association between TCR $_{\gamma/\delta}$ and CD3 chains. Non-reducing conditions were used for this immunoprecipitation to discriminate between the γ chain and its associated non-disulphide-linked δ chain. Antisera directed against the peptide from the predicted C_δ sequence again reacted with the 41 K δ chain protein for all three rabbits (Fig. 4e). The anti- γ chain peptide antiserum reacted with the diffuse 45K band, establishing it as a γ -chain product. These results indicate that the TCR cDNA in Pr 81 encodes the TCR $_\delta$ chain. The TCR $_\delta$ chain on these human cells is composed of a γ/δ heterodimer which is non-disulphide-linked, but some T cells express TCR $_{\gamma/\delta}$ which are disulphide-linked. The δ -chains in each type of heterodimer have quite distinct charge properties³⁰. Whether these chains are products of the same gene needs to be established to help our understanding of the function and specificity of cells expressing the TCR $_{\gamma/\delta}$.

We thank Dr Joe Phillips for providing TCR $_{\gamma/\delta}$ -bearing thymocytes, Dierdre Crommie, Marianne Newton, Robert Shields, Seve Cwirla and Andrew Sarafini for technical assistance, and Michael Armanini for help in the preparation of this manuscript. E.L. and A.W. are investigators of the Arthritis Foundation of America. M.M.D. is a scholar of the Pew Foundation. This work was supported in part by grants to M.M.D. from the National

Institutes of Health. The sequence data in this publication have been submitted to the EMBL/GenBank Libraries under the accession number Y00289.

Received 27 August; accepted 29 October 1987.

1. Schwartz, R. H. *A. Rev. Immun.* **3**, 237-261 (1985).
2. Livingstone, A. M. & Fathman, C. G. *A. Rev. Immun.* **5**, 477-501 (1987).
3. Babbitt, B. P., Allen, P. M., Matsueda, G., Haber, E. & Unanue, E. R. *Nature* **317**, 359-361 (1985).
4. Sette, A. *et al. Nature* **328**, 395-399 (1987).
5. Weiss, A. W. *et al. A. Rev. Immun.* **4**, 593-619 (1986).
6. Allison, J. P. & Lanier, L. L. *A. Rev. Immun.* **5**, 503-540 (1987).
7. Brenner, M. B. *et al. Nature* **322**, 145-149 (1986).
8. Bank, I. *et al. Nature* **322**, 179-181 (1986).
9. Weiss, A., Newton, M. & Crommie, D. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6998-7002 (1986).
10. Lanier, L. L. & Weiss, A. *Nature* **324**, 268-270 (1986).
11. Pardoll, D. M. *et al. Nature* **326**, 79-81 (1987).
12. Brenner, M. B. *et al. Nature* **325**, 689-694 (1987).
13. Borst, J. *et al. Nature* **325**, 683-688 (1987).
14. Lanier, L. L. *et al. J. exp. Med.* **165**, 1076-1094 (1987).
15. Davis, M. M. *A. Rev. Immun.* **3**, 537-560 (1985).
16. Kronenberg, M., Siu, G., Hood, L. E. & Shastri, N. *A. Rev. Immun.* **4**, 529-591 (1986).
17. Toyonaga, B. & Mak T. W. *A. Rev. Immun.* **5**, 585-620 (1987).
18. Chien, Y.-H., Iwashima, M., Kaplan, K. B., Elliott, J. F. & Davis, M. M. *Nature* **327**, 677-682 (1987).
19. Littman, D. R. *et al. Nature* **326**, 85-88 (1987).
20. Melton, D. A. *et al. Nucleic Acids Res.* **12**, 7035-7056 (1984).
21. Chen, E. Y. & Seeburg, P. H. *DNA* **4**, 165-170 (1985).
22. Tabor, S. & Richardson, C. C. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1074-1078 (1985).
23. Battula, N. & Loeb, L. A. *J. biol. Chem.* **249**, 4086-4093 (1974).
24. Gerard, G. F. *Focus* **8**, 30 (1986).
25. Sim, G. K. *et al. Nature* **312**, 771-775 (1984).
26. Kappler, J. *et al. Cell* **35**, 295-302 (1983).
27. Lindsten, T., Fowlkes, B. J., Samuelson, L., Davis, M. M. & Chien, Y. *J. exp. Med.* **166**, 761-765 (1987).
28. Phillips, J. H., Weiss, A., Gemlo, B. T., Raynor, A. A. & Lanier, L. L. *J. exp. Med.* (in the press).
29. Wall, R. & Kuehl, M. A. *Rev. Immun.* **1**, 393-422 (1983).
30. Lanier, L. L. *et al. J. clin. Immun.* (in the press).
31. Oetting, H. C., Kappler, J., Tax, W. J. M. & Terhorst, C. *J. biol. Chem.* **259**, 12039-12048 (1984).

Peptide sequences of T-cell receptor δ and γ chains are identical to predicted X and γ proteins

Willi Born*†, Craig Miles‡, Janice White‡, Rebecca O'Brien*, John H. Freed†‡, Philippa Marrack*§†, John Kappler*†‡|| & Ralph T. Kubo†‡||

* Howard Hughes Medical Institute, Denver, Colorado 80206, USA

‡ Department of Medicine, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado, USA

§ Departments of Biochemistry, Biophysics and Genetics,

† Microbiology and Immunology and || Medicine, University of Colorado Health Sciences Center, Denver, Colorado, USA

Although most mature peripheral T lymphocytes express a major histocompatibility complex restricted, CD3-associated, antigen receptor (TCR) which has been well characterized¹⁻¹⁶, some T cells carry a different CD3-associated heterodimer on their surface¹⁷⁻²². One of the two disulphide-linked chains of this putative second receptor, which in mice has relative molecular mass (M_r) 35,000 (35K), has been identified as a product of the group of γ genes¹⁸. The other chain, termed δ (M_r 45K in mice), is not as well characterized. Although γ/δ -bearing cells are a minor subset among peripheral T lymphocytes, they are the only CD3⁺ cells in the thymus early in ontogeny¹⁹. Taking advantage of these kinetics, we have generated γ/δ -bearing hybridomas, using a new TCR α chain-negative variant of the AKR thymoma BW5147 as tumour parent, fetal thymocytes as normal cell partners, and an anti-CD3 monoclonal antibody (mAb) as screening reagent²³. Gamma and δ chains from one of these hybrids have been purified and partially sequenced. The sequences obtained indicate that δ is indeed identical to the polypeptide encoded by the recently described gene X, as suggested by Chien *et al.*²⁴.

Fetal thymocytes from C57BL/6 mice at day 16 of gestation were fused to the new TCR α chain loss variant of BW5147 (generated by X-ray mutagenesis; J.W. *et al.*, manuscript in preparation). One hundred and three of the resulting hybridomas were tested for surface expression of the CD3 complex and nine were positive. None of these cells contained α/β receptor proteins precipitable with a pan-specific anti-TCR antiserum²⁵. Digitonin lysates of ¹²⁵I-surface-labelled cells were precipitated with a cross-reactive hamster anti-human CD3 mAb (HMT3-1; R.T.K. *et al.*, manuscript in preparation). Two-dimensional (unreduced/reduced) gel electrophoresis of the immunoprecipitates of one hybridoma, 33BTE-140, revealed a CD3-associated heterodimeric molecule composed of disulphide-bonded subunits with apparent relative molecular masses of 38K and 46K (Fig. 1a). The fact that this heterodimer was not precipitated with the pan-reactive anti-TCR antiserum suggested that it could consist of γ and δ chains. Therefore, 33BTE-140 cells were subcloned and one clone with particularly high CD3 surface expression, 33BTE-140.9, was chosen for further studies. Putative γ and δ chains of these cells were separated, purified by a modification of the two-dimensional unreduced/reduced SDS-PAGE procedure (Fig. 1b and ref. 16), and digested with trypsin. Two of the γ chain tryptic peptides, designated A and B, were sequenced (Fig. 2). The sequence of peptide A is identical in 9/9 identified amino acids to a portion of sequence unique to the mouse $V\gamma 4$ gene²⁶. The sequence of peptide B is identical to the 3' sequence predicted by $J\gamma 1$ and $J\gamma 4$ but not $J\gamma 2$ complementary DNA or genomic sequences^{27,28}. Thus, the γ chain protein isolated from 33BTE-140.9 is made up of $V\gamma 4$ and probably $J\gamma 1$ and $C\gamma 1$, indicating that these previously described γ genes do indeed encode expressed γ chains. This γ chain cannot be BW5147-derived as $V\gamma 3$ and $V\gamma 4$ are deleted in this tumour (D. Raulet, personal communication). Thus, the γ sequences we have obtained must be derived from the normal T-cell partner of 33BTE-140.9, a thymocyte present early in ontogeny. The fact that this cell expressed $V\gamma 4$ is not unexpected in view of the finding that

Fig. 1 Analysis of the CD3-associated heterodimer of 33BTE-140.9 and purification of γ and δ chains. *a*, Two-dimensional SDS-PAGE (dimension 1, non-reduced; dimension 2, reduced) of anti-CD3 immunoprecipitate prepared using mAb HMT3-1 (R.T.K. *et al.*, manuscript in preparation). 5×10^7 33BTE-140 cells were ^{125}I surface radiolabelled and lysed with digitonin³¹. The autoradiograph was exposed for 5 d. *b*, Two-dimensional SDS-PAGE analysis of a fraction (2.5×10^8 cells) of a digitonin lysate prepared using 10^{10} 33BTE-140.9 cells per batch incubated overnight with excess Sepharose 4B beads coupled with mAb HMT3-1. The bound $\gamma/\delta/\text{CD3}$ complex was eluted with 0.05M diethylamine, pH10, and immediately neutralized in a stream of CO_2 . Lyophilized eluates were resuspended in Laemmli sample buffer. The gel was stained with silver nitrate. For the isolation of γ and δ chains for sequencing, 50 μg cytochrome *c* were added to the lysate. The sample was then electrophoresed in two dimensions as described¹⁶. Bands containing the putative 46K δ chain and 38K γ chain were electroeluted using a CBS Scientific electroelution apparatus and a running buffer of 0.05M Tris acetate/0.1% SDS (8 h, 80 V, room temperature). Reduction of the eluted proteins was performed with 1 nmol of dithiothreitol (4 molar excess over estimated number of cysteine bounds) and alkylation with 4-vinylpyridine (16 molar excess). Subsequently, the running buffer was exchanged for 0.025 M ammonium bicarbonate during an additional 8 h at 50 V. Samples were lyophilized, and the remaining SDS removed from the protein by ion-pairing³². Samples were then dissolved in water and incubated 4 h with 5% w/w of TPKC-trypsin at room temperature. Lyophilized digests were redissolved in 0.1% trifluoroacetic acid (TFA) and loaded in 50- μl aliquots onto a 2.1 mm $\times$ 100 mm Aquapore RP-300 reverse phase column installed in an Applied Biosystems 120A high pressure liquid chromatograph. The column was eluted at a flow rate of 200 $\mu\text{l min}^{-1}$ with a linear gradient of 0.1% TFA in acetonitrile to 50% over 50 min at 45 $^\circ\text{C}$. The eluate was monitored at 214 nm and fractions were collected at 30-s intervals. The sequence of the peptides was determined by sequential degradation on an Applied Biosystems 470A protein microsequencer, using modified Edman chemistry.

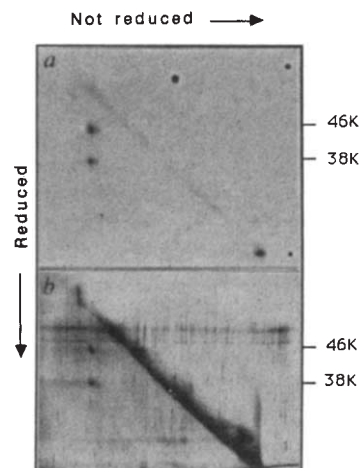
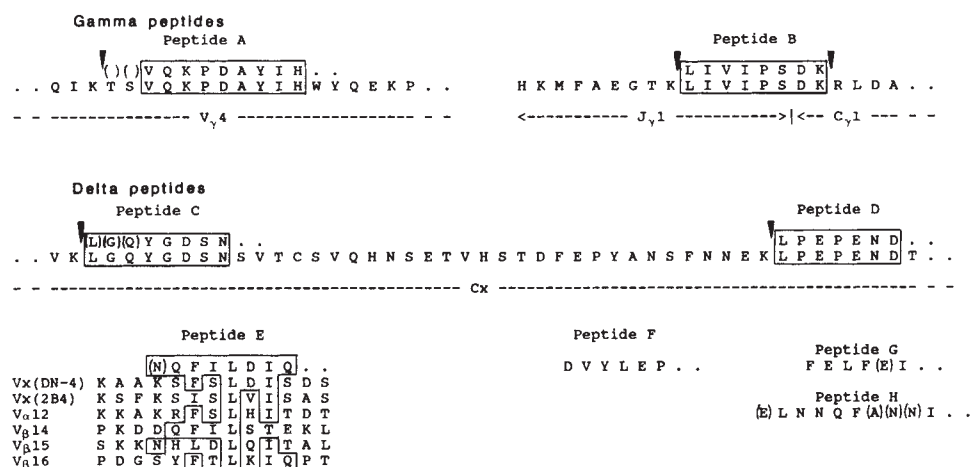


Fig. 2 Amino-acid sequences of the 33BTE-140.9-derived γ and δ tryptic peptides and comparison to the murine *X* gene and T-cell receptor α and β chain sequences. Peptides A and B are derived from the γ chain preparation (38K) and peptides C through H are derived from the δ -chain preparation (46K). Identical amino acids are boxed. Sequences shown for comparison are derived from the following sources: *V γ 1* and *V γ 4* and *J γ /C γ* (refs 26–28); *V α* , *J α* and *C α* sequences (ref. 24); *V α* and *V β* sequences (refs 33–35 and Yagüe *et al.*, manuscript in preparation); *C α* and *C β* sequences (refs 11, 13 and 14); *C μ* and *C λ* sequences (ref. 36). Throughout the paper, the designation of γ genes introduced by Garman *et al.*²⁶ has been followed. Assignments in parentheses indicate that more than one amino acid was found. Open parentheses indicate that no assignment could be made at that position. Arrows indicate predicted tryptic cleavage sites. Tryptic cleavage after the lysine in peptide A was probably inhibited by the adjacent proline residue³⁷.



V γ 4 and *V γ 3* are preferentially expressed at this early time of gestation²⁶.

The positive identification of γ peptides established that, by definition, its covalently bonded partner was a δ chain. Therefore, six tryptic peptides were sequenced from the purified δ protein (Fig. 2). Unambiguous sequences were obtained except for a few amino acids. Comparison of these peptides with T-cell receptor α and β chains and with the recently described *X* gene²⁴ showed extensive sequence similarities. The most striking was found for peptide D, which was identical in all seven amino acids to a constant region sequence of the *X* gene, whereas no matches were evident with other constant region sequences. The fact that this sequence is located in a portion of the *X* gene 5' to the third cysteine codon of the constant region, an area dissimilar to other T-cell receptor genes, strengthens the argument that the *X* gene encodes a δ polypeptide. Peptide C also showed similarities to the constant portion of *X* with at least 5/8 identical amino acids. This region of the corresponding *X* gene sequence, 5' to the second cysteine codon, is likewise dissimilar to other T-cell receptor genes. The first three amino acids in this peptide could not be identified unambiguously (Fig. 2). Three other amino acids (Ala, Asp, Val) were detected as

well as the amino acids predicted by the *X* gene sequence. Peptide F, found in a high pressure liquid chromatography fraction adjacent to peptide C, begins with Ala-Asp-Val; it is likely therefore that incomplete separation of these two peptides caused the ambiguity in the sequence of peptide C. Recently we have found by cDNA sequencing that the constant region of the 33BTE-140.9 δ gene is identical to *C α* , thus confirming that the possible amino-acid differences between peptide C and the *X*-gene sequence are artefactual (R.O'B. *et al.*, manuscript in preparation).

Peptide E is derived from the variable portion of 33BTE-140.9 δ . This eight amino-acid peptide shows equal similarities to *V α* and *V β* , including three highly conserved positions, and matching certain *V α* and *V β* sequences in all but one of the remaining five positions. The amino acid (Asp) in this position is, however, shared with *V α* . DNA sequence data confirm that this peptide identifies a new *V δ* gene. Similarly, peptide F has been identified as an N-terminal peptide of the same *V δ* gene (ref. 29 and D. Raulet, personal communication and R.O'B. *et al.*, manuscript in preparation). Peptides G and H are listed here for reasons of completeness. No obvious homologies have been found and, though these peptides could identify further δ chain sequences,

they are more probably derived from other contaminating proteins.

The sequences listed in Fig. 2 represent the first protein sequences reported for γ and δ chains. Although still fragmentary, our data allow several conclusions. First, the similarity of some of the peptide sequences with the predicted amino-acid sequence of the X gene should be noted. The matches to constant region sequences of this gene are perfect in all identified positions. Therefore, the X gene encodes a δ chain. As the M_r for 33BTE-140.9 δ (46K) is similar to other δ proteins described, the fact that the cDNA clone isolated by Chien *et al.*²⁴ predicts a polypeptide core of M_r 31K remains puzzling. Possible explanations have been previously discussed²⁴.

The X gene sequences are absent in the AKR thymoma line BW5147, presumably because of deletion during α gene rearrangement of both alleles³⁰. Therefore, 33BTE-140.9 expresses a δ chain which is derived from a normal, day-16 fetal thymocyte. The variable portion of this chain identifies a new V_δ gene which can be functionally expressed early in T-cell development.

In summary, the sequences confirm the immunoglobulin-like nature of the δ chain and suggest that δ and γ chains combine to create a diverse range of receptor molecules, similar to proteins encoded by immunoglobulin and TCR α and β genes. Thus, γ/δ heterodimers could function like α/β heterodimers on T cells, namely as receptors recognizing a diverse collection of ligands. Given that the percentage of γ/δ -bearing cells among all T cells is maximal early in gestation, at a time when normal exposure to antigen appears to be minimal, and occurs in immature thymocytes which apparently do not encounter foreign antigens, the present findings present an interesting puzzle. The solution could add to our understanding of T-cell differentiation and thymus function.

We thank Dr Jeffrey Bluestone for monoclonal anti-CD3 antibody, Terri Wade, Michele Pigeon and Judi Pasternak for technical help, and Inge Warvi and Donna J. Thompson for secretarial assistance.

Received 28 August; accepted 3 November 1987.

- Allison, P., McIntyre, B. W. & Bloch, D. J. *J. Immun.* **129**, 2293-2300 (1982).
- Meuer, S. *et al. J. exp. Med.* **157**, 705-719 (1983).
- Haskins, K. *et al. J. exp. Med.* **157**, 1149-1169 (1983).
- Kappler, J., Kubo, R., Haskins, K., White, J. & Marrack, P. *Cell* **34**, 727-737 (1983).
- Meuer, S. C. *et al. Nature* **303**, 808-810 (1983).
- Kappler, J. *et al. Cell* **35**, 295-302 (1983).
- McIntyre, B. W. & Allison, J. P. *Cell* **34**, 739-746 (1983).
- Acuto, O., Meuer, S. C., Hodgdon, J. C., Schlossman, S. F. & Reinherz, E. C. *J. exp. Med.* **158**, 1368-1373 (1983).
- Yanagi, Y. *et al. Nature* **308**, 145-149 (1984).
- Hedrick, S. M., Cohen, D. I., Nielsen, E. A. & Davis, M. M. *Nature* **308**, 149-153 (1984).
- Hedrick, S. M., Nielsen, E. A., Kavaler, J., Cohen, D. I. & Davis, M. M. *Nature* **308**, 153-158 (1984).
- Acuto, O. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 3851-3855 (1984).
- Chien, Y. *et al. Nature* **312**, 31-35 (1984).
- Saito, H. *et al. Nature* **312**, 36-40 (1984).
- Sim, G. K. *et al. Nature* **312**, 771-775 (1984).
- Hannum, C. H., Kappler, J. W., Trowbridge, I. S., Marrack, P. & Freed, J. H. *Nature* **312**, 65-67 (1984).
- Brenner, M. B. *et al. Nature* **322**, 145-149 (1986).
- Lew, A. M. *et al. Science* **234**, 1401-1405 (1986).
- Pardoll, D. M. *et al. Nature* **326**, 79-81 (1987).
- Bluestone, J. A., Pardoll, D. M., Sharrow, S. O. & Fowlkes, B. J. *Nature* **326**, 82-84 (1987).
- Borst, J. *et al. Nature* **325**, 683-688 (1987).
- Moingeon, P. *et al. Nature* **325**, 723-726 (1987).
- Leo, O., Foo, M., Sachs, D. H., Samelson, L. E. & Bluestone, J. A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1374-1378 (1987).
- Chien, Y., Iwashima, M., Kaplan, K. B., Elliott, J. F. & Davis, M. M. *Nature* **327**, 677-682 (1987).
- Kubo, R. T. & Roehm, N. *Molec. Immun.* **23**, 869-878 (1986).
- Garman, R. D., Doherty, P. J. & Raulet, D. H. *Cell* **45**, 733-742 (1986).
- Hayday, A. C. *et al. Cell* **40**, 259-269 (1985).
- Iwamoto, A. *et al. J. exp. Med.* **163**, 1203-1212 (1986).
- Chien, Y. *et al. Nature* (in the press).
- Lindsten, T., Fowlkes, B. J., Samelson, L. E., Davis, M. M. & Chien, Y. *J. exp. Med.* **166**, 761-775 (1987).
- Oettingen, H. C., Pettay, C. L., Maloy, W. L. & Terhorst, C. *Nature* **320**, 272-275 (1986).
- Konigsberg, W. H. & Henderson, L. *Meth. Enzym.* **91**, 254-259 (1983).
- Becker, D. M. *et al. Nature* **317**, 430-434 (1985).
- Barth, E. *et al. Nature* **316**, 517-523 (1984).
- Behlke, M. A., Chou, H. S., Huppi, K. & Loh, D. Y. *Proc. natn. Acad. Sci. U.S.A.* **83**, 767-771 (1986).
- Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. & Gottesman, K. S. *Sequences of Proteins of Immunological Interest* (US Dept Health and Human Services, 1987).
- Smyth, D. G. *Meth. Enzym.* **11**, 214-231 (1967).

Delta is the C_δ -gene product in the γ/δ antigen receptor of dendritic epidermal cells

Mark Bonyhadi*, Arthur Weiss†, Philip W. Tucker‡, Robert E. Tigelaar§ & James P. Allison*

* Cancer Research Laboratory and Department of Microbiology and Immunology, University of California, Berkeley, California 94720, USA

† Howard Hughes Medical Institute, University of California, San Francisco, California 94143, USA

‡ Department of Microbiology, University of Texas Southwestern Medical Center at Dallas, Texas 75235, USA

§ Department of Dermatology, University of Texas Southwestern Medical Center at Dallas, Texas 75235, USA

Most T cells bear an antigen receptor that is a protein of a disulphide-linked heterodimer composed of an α chain and a β chain associated with the non-polymorphic CD3 (T3) complex¹⁻⁷. A small subpopulation of thymic and peripheral T cells⁸⁻¹⁷, as well as Thy-1⁺ dendritic epidermal cells (dEC) (refs 18-21), express an alternative CD3-associated dimeric receptor composed of the product of the T-cell antigen receptor (TCR) γ gene²² and a fourth chain, designated δ . Recently a new murine TCR constant-region gene, designated C_δ , has been cloned and proposed as a candidate for the C_δ gene²³. We have previously demonstrated that murine Thy-1⁺ dEC cell lines express a CD3-associated disulphide-linked heterodimer composed of a relative molecular mass M_r 41,000 (41K) γ chain and a 50K δ chain²¹. We have further analysed the receptor of one of these cloned dEC lines, 7-17.1, by endoglycosidase treatment of the isolated γ and δ chains. The γ chain was found to contain two N-linked oligosaccharide residues, consistent with the expression of a chain encoded by the $V_{\gamma 3}$ and $C_{\gamma 1}$ gene segments. The δ chain contains at least three N-linked oligosaccharides and has a core size of 38K. Northern blot analysis indicated the presence of abundant C_δ messenger RNA in 7-17.1 cells. Immunoprecipitation with two antisera to peptides comprising distinct regions of the C_δ sequence indicates that the δ chain is encoded by the C_δ gene.

Thy-1⁺ dEC clone 7-17.1 was derived from the lymphokine dependent AKR/J cell line dEC 7-17 (ref. 21) by limit dilution cloning and maintained by feeding IL-2 every 3-4 days. Radio-

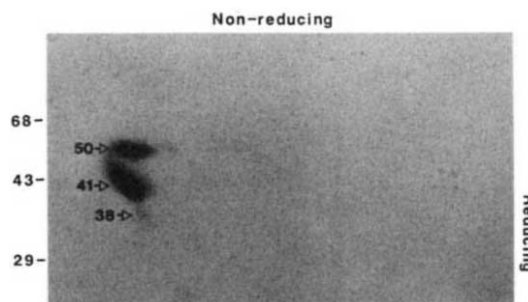


Fig. 1 Diagonal gel electrophoresis of CD3 associated γ/δ heterodimer of Thy-1⁺ dEC clone 7-17.1. Cells were radiolabelled¹ and solubilized in lysis buffer consisting of 5 mM CHAPS in 10 mM Tris-HCl, pH 8.0 and containing 1 mM phenylmethylsulphonyl fluoride, aprotinin, and 10 mM iodoacetamide²¹. Cell lysates were precleared with protein A-bearing *Staphylococcus aureus* coated with normal hamster serum (NHS). Immunoprecipitation was performed with hamster monoclonal antibody 500-A2 to murine CD3 (ref. 24). Diagonal electrophoresis was carried out on 10% polyacrylamide gels under non-reducing conditions, followed by SDS-PAGE on 12.5% gels under reducing conditions as previously described¹. Gels were dried and labelled proteins visualized by autoradiography at -70 °C with enhancing screens.

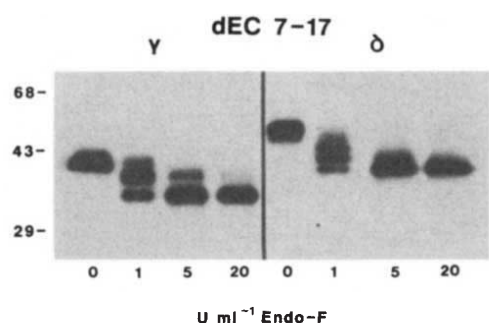


Fig. 2 Endoglycosidase digestion of γ and δ chains. CD3-associated γ and δ chains were isolated by radioimmunoprecipitation as described in the legend to Fig. 1. Immunoprecipitates were solubilized and subjected to one-dimensional SDS-PAGE on 11% gels as previously described¹. Gels were fixed for 30 min in 40% methanol/10% acetic acid, swollen for 15 min in 10% methanol/5% acetic acid/4% glycerol, and dried. γ and δ chains were located by autoradiography and excised. Gel slices were rehydrated in N-glyconase digestion buffer (0.17% SDS, 1.25% NP-40, 10 mM 2-mercaptoethanol in 0.2 M sodium phosphate buffer, pH 8.6), subjected to three cycles of freezing and thawing, and treated with 0, 1, 5 or 20 units ml^{-1} N-glyconase (Genzyme, Boston) for 16 h at 37 °C (ref. 30). The enzyme-treated gel slices were then boiled for 3 min in SDS-PAGE sample buffer containing 5% 2-mercaptoethanol and digested protein resolved by SDS-PAGE on a 2 cm stacking gel containing 4 M urea and an 11% resolving gel.

iodinated 7-17.1 cells were lysed in 5 mM 3-[(cholamidopropyl)-dimethylammonio]-2,2-hydroxyl-1 propane-sulphonate (CHAPS) to preserve the association between CD3 and its associated proteins. Immunoprecipitates were obtained with a monoclonal antibody to the ϵ chain of murine CD3 (ref. 24) and analysed by two-dimensional diagonal electrophoresis. As shown in Fig. 1, immunoprecipitates contained, in addition to the CD3 δ and CD3 ϵ chains, two major components of 50K and 41K, and a minor component of 38K. Using an antisera to a synthetic peptide comprising the C-terminal seven residues common to the murine $C_{\gamma 1}$, $C_{\gamma 2}$ and $C_{\gamma 3}$ genes, we have previously identified the 41K component as the γ chain²¹ and by convention have designated the 50K component as the δ chain. The location of the spots below the diagonal in Fig. 1 suggests that 7-17.1 cells express two forms of heterodimer, the major species composed of a δ chain associated with the 41K γ chain, and a minor species composed of a δ chain associated with the 38K component.

To further characterize the CD3-associated chains, SDS-PAGE slices containing the isolated bands were digested with graded quantities of N-glyconase to remove N-linked glycosyl residues in a stepwise manner. As shown in Fig. 2, the 40K γ chain yields products of 38K and 34K, indicating the presence of two N-linked oligosaccharide chains. This is consistent with our previous observation that 7-17 cells express $V_{\gamma 3}$ RNA (ref. 21). Although we cannot at present rule out strain-related polymorphism in glycosylation, the combination of $V_{\gamma 3}$ and $C_{\gamma 1}$ gene segments is the only γ gene combination that would yield a chain with two N-linked oligosaccharides^{25,26}. N-glyconase digestion of the 38K component (data not shown) yielded a single product of 34K, indicating the presence of a single N-linked oligosaccharide. This, together with the fact that the 38K band coprecipitates with an antiserum to $C_{\gamma 1-3}$ (Fig. 4, lane 4), suggests that it could be a glycosylation variant of the γ chain. The size of the fully digested chains is consistent with that expected from the complementary DNA sequence of γ genes.

N-glyconase treatment of the δ chain yielded a more complex pattern, consistent with the presence of a minimum of three N-linked oligosaccharides (Fig. 2). The fully digested chain was reduced to 38K, similar to the core size reported for other murine

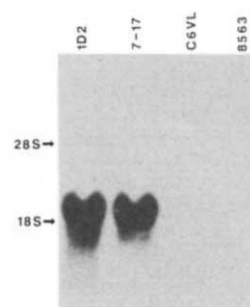


Fig. 3 Northern blot analysis of Thy-1⁺ dEC RNA with C_x . Total RNA (20 μg) from Thy-1⁺ dEC clones 1D2, 7-17.1, the T-lymphoma line C6VL, and the B cell myeloma 8653 were electrophoresed on a 1% agarose/formaldehyde gel, transferred on to nitrocellulose and baked. The blot was hybridized with C_x (ref. 23) in 1 M NaCl, 50 mM sodium phosphate, pH 6.5, containing 50% formamide, 100 $\mu\text{g ml}^{-1}$ salmon-sperm DNA, 4 $\times$ Denhardt's solution and 10% Dextran sulphate at 40 °C and washed in 2 $\times$ SSPE and 0.2 $\times$ SSPE at 55 °C (0.2 $\times$ SSPE is 2.98 M NaCl, 0.2 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.02 M EDTA). The positions of 28S and 18S ribosomal RNA are indicated.

δ chains^{14,23}. The core size of the C_x gene product, as predicted from the sequence of cDNA isolated with the C_x probe, is 31K (ref. 23). The discrepancy between this value and the size of the N-glyconase-treated δ chain suggested that C_x does not encode the δ chain. To determine whether murine Thy-1⁺dEC cells that expressed γ/δ receptors contained transcripts related to the C_x gene, RNA from 7-17.1 and 1D2 cells²¹ were analysed by Northern blots using a C_x probe²³. As shown in Fig. 3, both Thy-1⁺ dEC clones had an abundant C_x transcript of ~2 kilobases (kb). A T cell lymphoma, C6VL, which expresses an α/β heterodimer¹, and the myeloma 8653 lacked C_x transcripts. We further investigated the correlation of C_x -related RNA and expression of δ chain in the Thy-1⁺dEC cells.

Antisera were produced by immunization of rabbits with two peptides corresponding to different regions of the predicted C_x protein sequence (Fig. 4a). Peptide P014 corresponds to residues 216-231 of the murine C_x sequence²³. Peptide W6 corresponds to residues 159-187 of human C_x and residues 162-190 of the murine C_x gene product²⁷. The human and murine sequences are very similar in this region, differing only at residues 159, 174, 180 and 182. Antisera to the peptides were used in sequential immunoprecipitation to determine the origin of the CD3-associated proteins. Lysates of radioiodinated 7-17.1 cells were subjected to immunoprecipitation with anti-CD3, the associated proteins eluted with SDS, reduced and alkylated, renatured by dilution in NP-40 and reprecipitated with antisera to C_γ and C_x peptides. As shown in Fig. 4b, the antiserum to C_γ was reactive with the 41K chain. The antisera to the murine and human C_x peptides were both specifically reactive with the 51K δ chain. As the murine C_x peptide used for immunization contained a site for N-linked glycosylation, we also performed immunoprecipitation on anti-CD3 eluates which had previously been treated with N-glyconase. As shown in lane 6, this treatment greatly enhanced the reactivity of the anti-murine C_x serum, and yielded a band with the expected size of 37K. These results together are evidence that the CD3-associated δ chain is the product of the C_x gene.

The demonstration that murine Thy-1⁺ dEC cells express $V_{\gamma 3}$ transcripts is interesting because steady state levels of $V_{\gamma 3}$ RNA are high in 16-17 day fetal thymus, but $V_{\gamma 3}$ transcripts are not detectable in newborn or adult thymocytes of BALB/c mice^{25,26}. $V_{\gamma 3}$ expression has not been demonstrated in resting or activated peripheral T cells or in other T-cell lines^{25,26}. Our previous demonstration of $V_{\gamma 3}$ expression by four of four independently derived Thy-1⁺ dEC lines from AKR/J mice suggests either a bias in this strain for $V_{\gamma 3}$ usage, or, alternatively that the $V_{\gamma 3}$

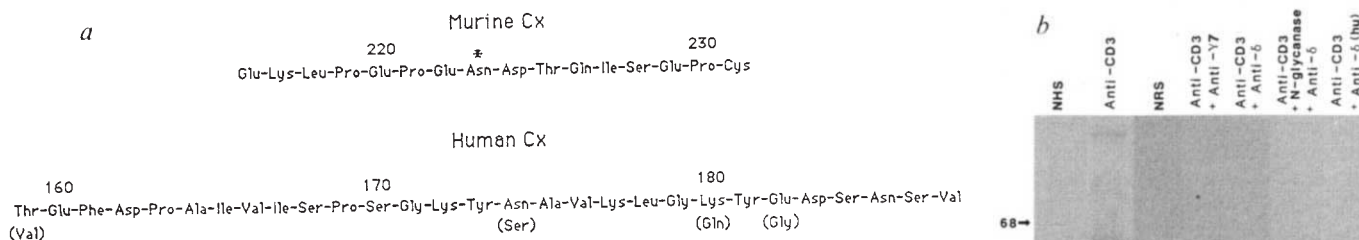


Fig. 4 Immunoprecipitation of CD3-associated γ and δ chains by anti-peptide antisera. **a**, Sequence of C_x peptides. Antisera were produced by immunization of rabbits with synthetic peptides comprising the indicated sequences of the murine (P014) or human (AW06) C_x sequences. The asterisk indicates a site for potential N-glycosylation. **b**, Immunoprecipitation analysis. Thy-1⁺ dEC 7-17.1 cells were radioiodinated and lysates prepared using CHAPS as described in the legend for Fig. 1. Immunoprecipitates were obtained with normal hamster serum (lane 1) or with anti-CD3 antibody 500-A2 (lane 2) (ref. 24). For sequential immunoprecipitation, CD3-associated components were eluted by treatment with sample buffer containing 0.1% SDS and 2 mM dithiothreitol for 15 min at 68°C, followed by alkylation with iodoacetamide (15 mM) and dilution with four volumes 1.5% NP-40 in 0.01 M Tris-HCl, pH 8.0. After 1 h at 4°C, samples were precipitated with normal rabbit serum (NRS) (lane 3), an antiserum to a heptapeptide common to C γ 1-3 (lane 4), an antiserum to a murine C_x peptide (lanes 5 and 6), or an antiserum to a human C_x peptide (lane 7). The sample in lane 6 was digested with N-glycanase (2 U ml⁻¹) for 12 h at 37°C immediately before precipitation with anti-murine C_x.

gene is preferentially used in Thy-1⁺ dEC cells. This latter possibility suggests that Thy-1⁺ dEC cells could represent very early emigrants from the developing thymus. The thymic origin of Thy-1⁺ dEC cells has not been definitely demonstrated, but the apparent bias for lineage-specific useage of the V γ 3 gene has implications for the origin and function of these cells.

The fact that C_x codes for the antigen receptor δ chain has important implications for thymic differentiation. It has been established that the J α C_x locus rearranges and is expressed before the J α C_α locus, and that the J α C_x locus is almost always biallelically deleted in cells that express α (ref. 23). The location of the C_x locus between V α and J α ensures that rearrangement of V α to J α will lead to deletion of the C_x locus. This allows for at least two alternative models for the influence of antigen receptor gene rearrangement on the lineage of T cells. One possibility is that immature T cells express a receptor composed of the products of successfully rearranged γ and δ genes, and then mature into cells that express α/β receptors^{25,28}. A second possibility is that early and successful in-frame rearrangement of both the γ and δ loci prevents subsequent rearrangement of the β and α loci, thus committing cells to the γ/δ pathway. Cells which fail to successfully rearrange either or both γ and δ continue to rearrange β and α with the concomitant deletion of the δ locus. Those cells that successfully rearrange both the α and β loci undergo thymic selection and could ultimately give rise to mature α/β T cells. This model predicts separate and independent lineages for γ/δ and α/β T cells from a common precursor^{14,29}. In either model, if allelic exclusion is operative, the location of the δ locus within α provides a mechanism for ensuring that a given T cell expresses only a single receptor, composed of either α/β or γ/δ .

We thank Dr Y.-H. Chien for providing the C_x probe before publication and for the Northern blot analysis. This research was supported by the NIH.

Received 7 September; accepted 3 November 1987.

- Allison, J. P., McIntyre, B. W. & Bloch, D. J. *Immun.* **129**, 2293-2300 (1982).
- Haskins, K. et al. *J. exp. Med.* **157**, 1149-1169 (1983).
- Meuer, S. et al. *J. exp. Med.* **157**, 705-719 (1983).
- Borst, J., Alexander, S., Elder, J. & Terhorst, C. *J. biol. Chem.* **258**, 5135-5143 (1983).
- Allison, J. P. & Lanier, L. L. *Nature* **314**, 107-108 (1985).
- Samuelson, L. E., Harford, J. B. & Klausner, R. D. *Cell* **43**, 223-231 (1985).
- Oettingen, H. C., Pettey, C. L., Maloy, W. L. & Terhorst, C. *Nature* **320**, 272-275 (1986).
- Bank, I. et al. *Nature* **322**, 179-181 (1986).
- Brenner, M. B. et al. *Nature* **322**, 145-149 (1986).
- Weiss, A., Newton, M. & Crommie, D. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6998-7002 (1986).
- Borst, J. et al. *Nature* **325**, 683-688 (1987).
- Moingeon, P. et al. *Nature* **325**, 723-726 (1987).
- Lew, A. M. et al. *Science* **234**, 1401-1405 (1986).
- Pardoll, D. et al. *Nature* **326**, 79-81 (1987).
- Bluestone, J. A., Pardoll, D., Sharrow, S. O. & Fowlkes, B. J. *Nature* **326**, 82-84 (1987).

- Brenner, M. B. et al. *Nature* **325**, 689-694 (1987).
- Lanier, L. L. et al. *J. exp. Med.* **165**, 1076-1094 (1987).
- Koning, F. et al. *Science* **236**, 834-837 (1987).
- Yokoyama, W. M. et al. *J. exp. Med.* **165**, 1725-1730 (1987).
- Stingl, G. et al. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2430-2434 (1987).
- Kuziel, W. A. et al. *Nature* **328**, 263-266 (1987).
- Saito, H. et al. *Nature* **309**, 757-762 (1984).
- Chien, Y.-H., Iwashima, M., Kaplan, K., Elliott, J. F. & Davis, M. M. *Nature* **327**, 677-682 (1987).
- Allison, J. P. et al. in *The T Cell Receptor, UCLA Symp. on Molecular and Cellular Biology* (eds Kappler, J. & Davis, M.) (Liss, New York, 1987).
- Garman, R. D., Doherty, P. J. & Raulet, D. H. *Cell* **45**, 733-742 (1986).
- Heilig, J. S. & Tonegawa, S. *Nature* **322**, 836-840 (1986).
- Loh, E. Y. et al. *Nature* (in the press).
- Raulet, D. H., Garman, R. D., Saito, H. & Tonegawa, S. *Nature* **314**, 103-107 (1985).
- Allison, J. P. & Lanier, L. L. *Immun. Today* **8**, 293-296 (1987).
- Rosenzweig, S. A., Madison, L. D. & Jamieson, J. D. *J. Cell Biol.* **99**, 1110-1116 (1984).

Targetted correction of a mutant HPRT gene in mouse embryonic stem cells

Thomas Doetschman*, Ronald G. Gregg*, Nobuyo Maeda*, Martin L. Hooper†, David W. Melton‡, Simon Thompson‡ & Oliver Smithies*§

* Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706, USA

† Department of Pathology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, UK

‡ Department of Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JR, UK

Two recent developments suggest a route to predetermined alterations in mammalian germlines. These are, first, the characterization of mouse embryonic stem (ES) cells¹ that can still enter the germline after genetic manipulation in culture^{2,3} and second, the demonstration that homologous recombination between a native target chromosomal gene and exogenous DNA can be used in culture to modify specifically the target locus⁴. We here use gene targetting functionally to correct the mutant hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene in the ES cell line which has previously been isolated and used to produce an HPRT-deficient mouse⁵. This modification of a chosen gene in pluripotent ES cells demonstrates the feasibility of this route to manipulating mammalian genomes in predetermined ways.

Three male ES cell lines in which the X-linked HPRT gene

§ To whom correspondence should be addressed.

Fig. 1 Planned correction of a deletion mutation in the HPRT gene of ES cells. *a*, The normal mouse HPRT gene¹⁸ showing its nine exons (not to scale) and the spontaneous deletion (dashed, thin lines) that produced the HPRT⁻ mutation in the ES cell line, E14TG2a (see ref. 5). The interrupted heavy line is DNA an undetermined distance 5' to the HPRT locus. *b*, The HPRT locus in the deletion mutant E14TG2a. H shows an *Hind*III site used in mapping. (H) shows an unmapped site. The (+) sign shows an *Hind*III site present in the chromosome but absent in the correcting plasmid. *c*, The 12.4-kb plasmid (pNMR133), used to correct the deletion via homologous recombination, was cut in exon 3, as shown, before introduction into the ES cells. The open box represents human sequences, the heavy line, mouse sequences and the continuous thin line, pAT153 sequences (not to scale). The (-) sign shows where the (+) *Hind*III site was removed by the insertion of 4 bp during construction of the plasmid. *d*, The HPRT locus after functional correction by homologous recombination. The corrected locus can have any combination of the two (+) or (-) sites depending on the occurrence of branch migration, heteroduplex repair, and exolytic degradation with gap repair during the recombination (see ref. 19) and on where crossovers resolve. *e*, The predicted sizes and locations of *Hind*III fragments expected to hybridize to an exon 3-specific probe with DNA from the HPRT⁻ mutant and from corrected HPRT⁺ cells having the indicated combinations of the (+) or (-) sites. The asterisks indicate fragments predicted to hybridize also to a probe specific for the plasmid vector. The uncertainty in two predicted sizes stems from lack of knowledge of the location of the (H) site.

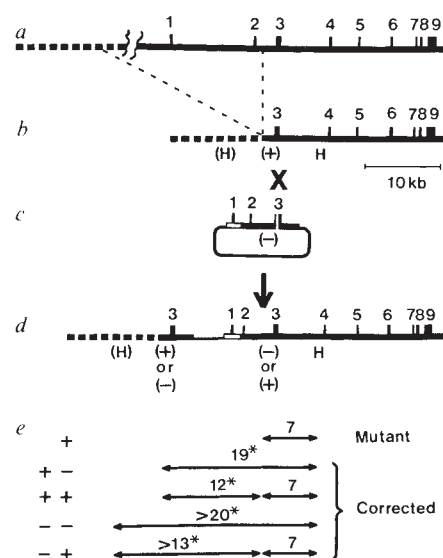
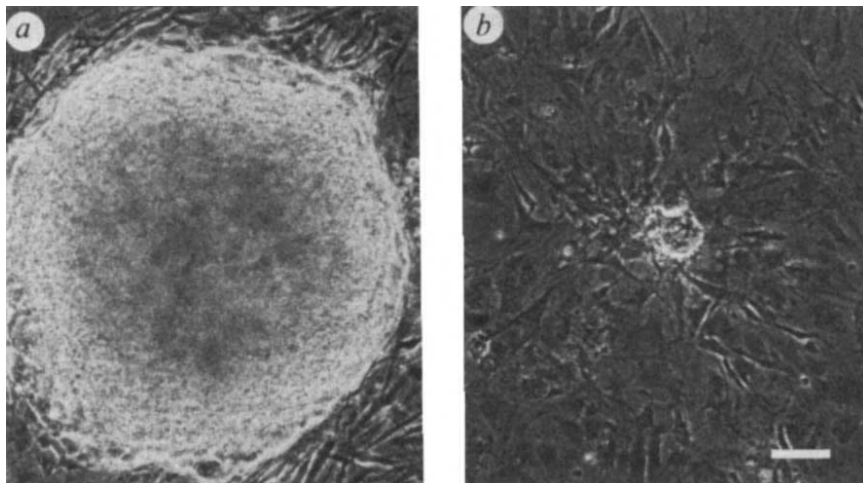


Fig. 2 ES cell colonies, growing on primary embryonic fibroblasts in the presence of HAT, 14 days after electroporation of HPRT⁻ ES cells in the presence of the correcting plasmid. *a*, A large colony scored as HPRT⁺ (see text). *b*, A small colony scored as HPRT⁻. Scale bar, 100 μ m.



is inactive have been isolated and used to produce mice that transmit the mutant genes to their progeny^{5,6}. The inactive HPRT gene in the ES cell line E14TG2a (see ref. 5) is due to a spontaneous deletion extending from at least 10 kilobases (kb) 5' to the promoter of the gene into its second intron (S.T., D.W.M. and M.L.H., unpublished data). The two other inactive HPRT genes in ES cells⁶ resulted from integration of retroviral sequences into different introns of the gene. The value of introducing mutations of specific genes, such as these obtained by random procedures, into the germline of experimental animals is self-evident. But its usefulness would be greatly enhanced if it could be targeted to the locus of interest so as to modify the DNA in a predetermined way. Homologous recombination between a target chromosomal locus and exogenous DNA having sequences in common with the target⁷⁻¹⁴ has this potential, and it can be used with a native chromosomal gene⁴.

We have now used homologous recombination with exogenous DNA functionally to correct the deletion in the HPRT gene of the ES cell line E14TG2a by supplying human and mouse sequences to replace the deleted promoter, exon 1 and exon 2 (see Fig. 1). The correcting plasmid, pNMR133, has between 2.5 and 5 kb of DNA in common with the target locus (more precise definition of the length awaits determination of the exact endpoint of the deletion). The recombination, a single crossover initiated at a pre-cut *Xho*I site in exon 3 within the common region, was planned to insert the 12.4-kb plasmid into the mutant HPRT locus of the ES cell and restore its function.

Linearized plasmid DNA was introduced into the HPRT⁻ ES cells by electroporation¹⁵. The treated cells were plated onto irradiated HPRT⁺ mouse embryonic fibroblast feeder cells and exposed to HAT (hypoxanthine, aminopterin, thymidine)-containing medium to select for HPRT⁺ ES cells. After 10-14 days of culture we observed two types of ES-cell colonies on the feeder layers (see Fig. 2). The small colonies, composed of a few cells, slowly died. We interpret them as HPRT⁻ colonies whose survival is prolonged by transfer of metabolites from the feeder cells. The large colonies, composed of thousands of cells, continued to grow on the feeder layer, and also when placed in HAT-containing medium conditioned by buffalo rat liver cells¹⁶. We score them as HPRT⁺. They were not seen when electroporation was omitted or was carried out in the absence of the correcting plasmid.

To determine whether the HPRT⁺ colonies were correctly modified by gene targeting, we analysed the DNA from five independent colonies by Southern blotting and compared their patterns after *Hind*III digestion with those predicted (see Fig. 1). The results are shown in Fig. 3. The autoradiograph with the HPRT gene-specific probe (Fig. 3a) shows that all five colonies give bands corresponding to correctly modified genes. Two colonies have the (+)(-) combination of *Hind*III sites expected from a simple crossover (see Fig. 1) and three have the (+)(+) combination of sites expected to result from crossing over accompanied by gap repair or gene conversion. Only one of the bands in each colony is also detected with a probe specific for

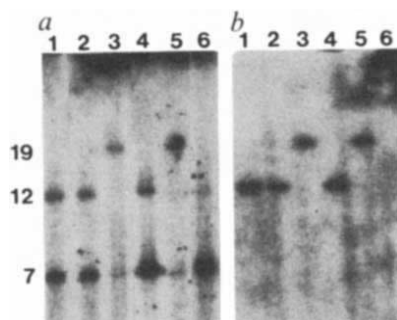


Fig. 3 Southern blot analysis of *Hind*III digests of genomic DNA from HPRT⁺ and HPRT⁻ ES cell colonies. *a*, A blot hybridized to a cDNA probe containing exons 3, 4 and 5 of the human HPRT gene. This probe is functionally specific for exon 3 of the mouse HPRT gene under our hybridization conditions. *b*, A second blot hybridized to a probe specific for the vector plasmid. Lanes 1, 2 and 4 contain DNA from HPRT⁺ ES cell colonies with the (+)(+) combination of *Hind*III sites (see Fig. 1), lanes 3 and 5 HPRT⁺ colonies with the (+)(-) combination of *Hind*III sites, lane 6, the starting HPRT⁻ ES cell line, E14TG2a. Measured fragment sizes are shown in kb. (Note: the 7 kb band faintly hybridizing to the HPRT-specific probe in lanes 3 and 5 is due to unavoidable contamination of the DNA from ES cells with DNA from the non-mutant feeder cells.)

the plasmid vector (Fig. 3*b*), establishing that the plasmid integrated solely into the HPRT locus. Thus the HPRT⁻ mutation is corrected in the predicted way in these HPRT⁺ colonies by plasmid sequences that integrate only into the target locus.

The average frequency of functional correction of the HPRT⁻ gene in three experiments (Table 1) was 1.4×10^{-6} per treated cell. The frequency at which we obtain G418-resistant colonies after electroporation of ES cells under comparable conditions in the presence of pSV2Neo¹⁷ ranges from 3×10^{-5} to 6×10^{-6} with a mean of about 1×10^{-5} . The frequency of targeted correction of the HPRT⁻ gene in the present experiments is therefore ~14% of the frequency of obtaining G418-resistant colonies. (Note, however, that the SV40 promoter in pSV2Neo probably only functions in a limited number of locations in the ES cell genome, whereas the corrected HPRT genes should be expressed in every cell.) Thus the route of gene targeting in ES cells shows considerable promise as a practical means of obtaining specific, predetermined germline changes in experimental animals. Gene targeting has also been used by Capecchi and his collaborators (personal communication) and by us (T.D. *et al.*, in preparation) to create HPRT⁻ mutants in ES cells. We are now attempting

Table 1 Targeted correction of HPRT⁻ embryonic stem cells

Number of treated cells ($\times 10^{-7}$)	DNA concentration (nM)	Electroporation (V cm ⁻¹)	HAT-resistant colonies	Frequency* ($\times 10^6$)
4	5	1,000	15	0.38
10	5	1,200	270	2.7
4.5	2.5	1,200	48	1.1

The HPRT⁻ embryonic stem cells (E14TG2a)⁵ were electroporated as described previously¹⁵ in the presence of the correcting plasmid, pNMR133, linearized at the *Xho*I site in exon 3 of its HPRT gene sequences. 5×10^6 Treated cells were plated per 100 mm dish of irradiated HPRT⁺ primary mouse embryonic fibroblasts in high glucose DMEM medium containing 15% fetal bovine serum and 0.1 mM 2-mercaptoethanol. The medium was replaced 24 h after electroporation with selective medium containing HAT. Colonies were scored as HAT-resistant if they were large (see Fig. 2*a*) after 14 days.

$$* \text{ Frequency} = \frac{\text{HAT-resistant colonies}}{\text{Number of treated cells}}$$

to increase the generality of gene targeting by extending it to genes that do not have selectable phenotypes and by avoiding the introduction of any foreign DNA into the target locus.

We thank Natalie Borenstein for tissue culture, Mike Ganske for electroporating ES cells and Dr Hyung-Suk Kim for some Southern blots. This work was supported by the NIH, Cancer Research Campaign and the Medical Research Council.

Received 30 September; accepted 27 October 1987.

- Evans, M. J. & Kaufman, M. H. *Nature* **292**, 154-156 (1981).
- Gossler, A., Doetschman, T., Korn, R., Serfling, E. & Kemler, R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9065-9069 (1986).
- Robertson, E., Bradley, A., Kuehn, M. & Evans, M. *Nature* **323**, 445-448 (1986).
- Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A. & Kucherlapati, R. S. *Nature* **317**, 230-234 (1985).
- Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. *Nature* **326**, 292-295 (1987).
- Kuehn, M. R., Bradley, A., Robertson, E. J. & Evans, M. J. *Nature* **326**, 295-298 (1987).
- Smith, A. J. H. & Berg, P. *Cold Spring Harb. Symp. quant. Biol.* **149**, 171-181 (1984).
- Smithies, O., Koralewski, M. A., Song, K.-Y. & Kucherlapati, R. S. *Cold Spring Harb. Symp. quant. Biol.* **49**, 161-170 (1984).
- Lin, F.-L., Sperle, K. K. & Sternberg, N. *Cold Spring Harb. Symp. quant. Biol.* **49**, 139-149 (1984).
- Lin, F.-L., Sperle, K. & Sternberg, N. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1391-1395 (1985).
- Rubnitz, J. & Subramani, S. *Molec. cell. Biol.* **6**, 1608-1614 (1986).
- Thomas, K. R., Folger, K. R. & Capecchi, M. R. *Cell* **44**, 419-428 (1986).
- Gregg, R. G. & Smithies, O. *Cold Spring Harbor Symp. quant. Biol.* **51**, 1093-1099 (1986).
- Song, K.-Y., Schwartz, F., Maeda, N., Smithies, O. & Kucherlapati, R. S. *Proc. natn. Acad. Sci. U.S.A.* **84**, 6820-6824 (1987).
- Boggs, S. S., Gregg, R. G., Borenstein, N. & Smithies, O. *Expl. Hemat.* **14**, 988-994 (1986).
- Smith, A. G. & Hooper, M. L. *Dev. Biol.* **121**, 1-9 (1987).
- Southern, P. J. & Berg, P. *J. Molec. Appl. Genet.* **1**, 327-341 (1982).
- Melton, D. W., Konecki, D. S., Brennand, J. & Caskey, C. T. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2147-2151 (1984).
- Ayares, D., Ganca, D., Chekuri, L., Campbell, C. R. & Kucherlapati, R. *Molec. cell. Biol.* **7**, 1656-1662 (1987).

Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer

Klaas Kok*, Jan Osinga*, Ben Carritt†, Mary B. Davis‡, Annemarie H. van der Hout*, Anneke Y. van der Veen*, Rudy M. Landsvater*, Lou F. M. H. de Leij§, Henk H. Berendsen||, Pieter E. Postmus||, Sibrand Poppema¶ & Charles H. C. M. Buys*#

* Department of Human Genetics, State University of Groningen, Groningen, The Netherlands

† MRC Human Biochemical Genetics Unit, University College, London, UK

‡ Charing Cross and Westminster Hospital Medical School, London, UK

§ Department of Clinical Immunology, State University of Groningen, Groningen, The Netherlands

|| Department of Pulmonary Diseases, State University of Groningen, Groningen, The Netherlands

¶ Department of Pathology, State University of Groningen, Groningen, The Netherlands

In childhood malignancies such as retinoblastoma and Wilms tumour, of which both familial and sporadic forms exist, recessive mutations of presumed differentiation genes have been implicated in tumorigenesis^{1,2}. A proportion of cases appear with microscopically visible chromosome deletions which indicate the regions where the genes concerned are located. Mutation or loss of one allele causes a cancer predisposition. For tumour development functional loss of the remaining normal allele is also required. In cancers with both familial and sporadic forms, molecular-genetic studies have shown that deletion is often one of the mutational events²⁻⁵.

To whom correspondence should be addressed.

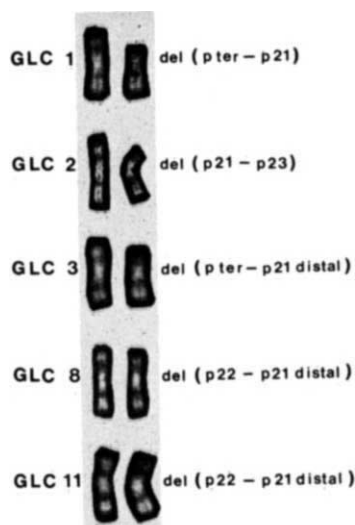


Fig. 1 Pairs of morphologically normal (left) and abnormal (right) chromosome-3 homologues from five Groningen lung cancer (GLC) cell lines derived from different SCLC tumours. The total number of chromosomes per cell varied between 51 and 136. In some metaphases more than two copies of chromosome 3 were present, always including at least one morphologically-normal and one abnormal homologue. All abnormal homologues show short arm deletions. The extent of each deletion is described at right. The shortest region of overlap of these deletions is 2p21 distal-p22. **Methods.** Chromosome spreads were prepared according to routine methods. Chromosomes were banded by double staining with DAPI and daunomycin²⁸.

Although familial and sporadic forms have never been distinguished in lung cancer, deletions of the short arm of chromosome 3 have been described for small cell lung cancer (SCLC) (refs 6, 7), but their general occurrence in SCLC has been disputed⁸⁻¹⁰. Using a molecular-genetic approach, we here present evidence for a consistent deletion at the chromosomal region 3p21, not only in SCLC¹¹, but in all major types of lung cancer.

Analysis of five SCLC cell lines revealed the presence of at least one morphologically normal and one deleted homologue of chromosome 3 per cell line. The shortest region of overlap for the deletion originally reported as 3p14-3p23 (ref. 6), could be narrowed to 3p21 distal-p22 (Fig. 1). A human DNA clone, λ Ch4A-H3 (D1S1), previously mapped to 1p by *in situ* hybridization¹², has recently been shown to originate from chromosome 3 (refs 13, 14). This may explain why after *in situ* hybridization grains were found not only over 1p36, but to a minor degree also over 3p21 (ref. 12). A subclone, pH3H2, detects a restriction fragment length polymorphism (RFLP) on chromosome 3 with *Hind*III fragments of 2.3 kilobase (kb) and 2.0 kb in addition to constant fragments of 8.0 kb and 3.8 kb from chromosome 1 (ref. 13). To localize the polymorphism unambiguously, the neighbouring subclones pH3E4 and pH3E4.8, limited to chromosome 3 (ref. 12), were used for *in situ* hybridization. Figure 2 shows their mapping to 3p21. A Southern analysis of cell lines derived from tumours of 20 different SCLC patients revealed only a single hybridizing chromosome-3-specific *Hind*III fragment in all cases. Taking into account a heterozygote frequency of 0.5 for the H3H2 polymorphism^{14,15}, the probability of finding homozygosity for 20 constitutive DNA samples as a random effect is less than 10^{-6} ($0.5 \exp 20$). Thus, loss of one allele at 3p21 in tumour cells in comparison with normal cells seems very likely for at least some patients. This conclusion was substantiated by analysing combinations of tumour material and leukocytes. Of seven patients found to be heterozygous with pH3H2 in their leukocyte DNA, tumour material was also available. In all cases the tumour DNA was fully homozygous

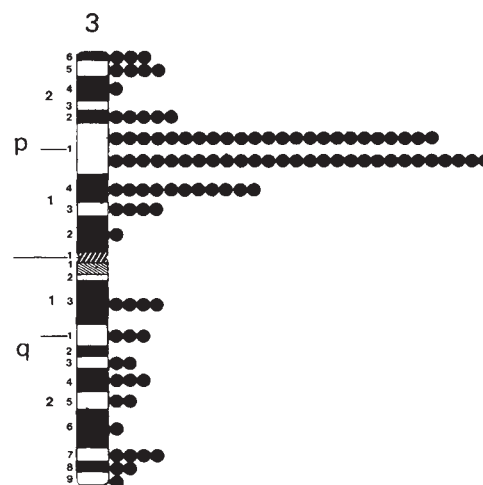


Fig. 2 Regional distribution of silver grains on chromosome 3 following *in situ* hybridization of normal lymphocyte metaphases with subclones adjacent to H3H2. Out of 289 grains scored in 100 metaphases 63 were on chromosome 3. After scoring an additional 40 chromosome-3 with a grain, a total of 103 labelled chromosomes 3 resulted. About half of the grains were in band p21.

Methods. *In situ* hybridization was carried out essentially according to ref. 29. To preserve a better chromosome morphology and thus obtain a better banding pattern, chromosomal DNA was denatured by alkaline treatment (4 min in 150 mM NaOH in 70% ethanol) instead of applying heat denaturation in 70% formamide.

or hemizygous for either the 2.3-kb or the 2.0-kb fragment (see examples in Fig. 3).

From a series of 16 biopsies taken from squamous cell carcinoma of the lung, *Hind*III-digested DNA was subjected to Southern analysis with pH3H2. The autoradiographs showed single bands in 12 cases, whereas in the remaining four cases two bands were visible, one being much fainter than the other. This apparent heterozygosity might be explained by a significant admixture of normal cells in the biopsy, as is often the case in non-SCLC. Therefore, autoradiographs were analysed by densitometry. Heterozygous patterns of 14 leukocyte and placental DNA samples scanned as normal controls, gave relative intensities of the 2.3-kb and 2.0-kb bands between 0.9 and 1.2. From Table 1 it is clear that the two bands in the autoradiographs from the squamous lung cancer samples have significantly different intensities. This indicates that the fainter band must be due to cellular heterogeneity, most probably between tumour and normal cells present in the same sample. We conclude that these samples do contain tumour cells which have lost one allele at 3p21. We infer that the same applies to at least some of the 12 cases showing only a single band. This conclusion is corroborated by finding complete loss of heterozygosity in tumours obtained from two patients from whom both constitutive and tumour DNA were available (Fig. 3).

The results of using pH3H2 in a Southern analysis of 14 adenocarcinoma samples are included in Table 1. Specimens of this type frequently have a larger admixture of normal cells. Consequently, we found a sizeable proportion of tumours producing two bands, again markedly differing in intensity. According to the above reasoning they must contain tumour cells which have lost one allele at 3p21. From five of these cases we could also collect blood samples. The leukocyte DNA proved to be clearly heterozygous (see Fig. 3).

Only one large cell carcinoma was available for analysis. The DNA gave two bands on the autoradiograph with an intensity ratio far outside the control range (Table 1). This can be taken as evidence for loss of one allele in a majority of cells in this specimen.

For the tumours that appeared with unequal intensities of the

Table 1 Alleles at 3p21 in non-SCLC

Tumour no.	Origin	Alleles present	Intensity ratio (allele 1/allele 2)
85-11956	Squamous cell carcinoma metastasis lymph node cervical	1	
86-4657	lobectomy	1	
86-4731	lobectomy	1	
86-5181	lobectomy	1, 2	0.6
86-5309	lobectomy	2	
86-6061	lobectomy	1	
86-6241	lobectomy	2	
86-6366	lobectomy	1	
86-6505	pneumonectomy	1	
86-6616	lobectomy	2	
86-6816	bilobectomy	2	
86-8123	lobectomy	2	
86-8574	pneumonectomy	1, 2	2.8
86-8826	wedge excision	1, 2	2.4
86-8777	metastasis hilar lymph node	2	
86-8869	pneumonectomy	1, 2	2.6
85-2553	Adenocarcinoma pneumonectomy	2	
85-3649	lobectomy	1, 2	0.4
85-4322	lobectomy	1, 2	0.5
85-7420	lobectomy	2	
85-10968	pneumonectomy	2	
85-11619	metastasis in dermis	2	
86-1501	metastasis lymph node supraclavicular	1	
86-4849	pneumonectomy	2	
86-5602	lobectomy	1, 2	2.1
86-5680	lobectomy	1, 2	1.5
85-7063	metastasis paratracheal	1, 2	1.8
86-8718	lobectomy	1, 2	0.6
86-9298	lobectomy	1	
86-9309	lobectomy	1, 2	0.3
86-621	Large cell carcinoma pneumonectomy	1, 2	0.2

The presence of alleles 1 (2.3 kb) and 2 (2.0 kb) recognized by pH3H2 was determined by Southern hybridization as described in the legend to Fig. 3. Autoradiographs were scanned with a Beckman R112 scanning densitometer. A diagnosis of squamous cell carcinoma was based on the presence of keratin pearls or intercellular bridges, or both. The diagnosis was confirmed by immunohistological analysis, indicating staining for cytokeratin 10 with monoclonal antibody RKSE 60 (ref. 26) in part of the tumour cells. A diagnosis of adenocarcinoma was based on the presence of tumour cells forming glands or intracellular mucus in more than a trace amount. The diagnosis was confirmed by immunohistological analysis, indicating the strong staining for cytokeratin 18 with monoclonal antibody RGE 53 in all tumour cells. A diagnosis of small cell carcinoma (not included in this table) was based on the presence of tumour cells with relatively small nuclei ($<25\ \mu\text{m}$) and lacking squamous or glandular differentiation. The diagnosis was confirmed by immunohistological staining with monoclonal antibody Moc 1 (ref. 27). A diagnosis of large cell carcinoma was made by exclusion. The tumour cells had large nuclei ($>25\ \mu\text{m}$) and lacked squamous or glandular differentiation.

2.3-kb and 2.0-kb bands, in principle the alternative explanation of unequal copy numbers of complete homologues exists. This was ruled out, however, by hybridizing five such specimens (three squamous and two adenocarcinomas) and the corresponding blood samples with both a 3p probe (pH3E4 at 3p21) and a 3q probe (DR2 at 3q21-qter (ref. 16)). Compared to the blood samples, the 3p signal in the tumours was much fainter ($37\% \pm 19\%$) than the 3q signal.

Rehybridization of filters containing *Hind*III-restricted DNA from a collection of all types of lung cancer with p9A7 detecting a *Hind*III polymorphism at 13q22-qter, showed heterozygous patterns in 14 of 28 tumours in agreement with this probe's

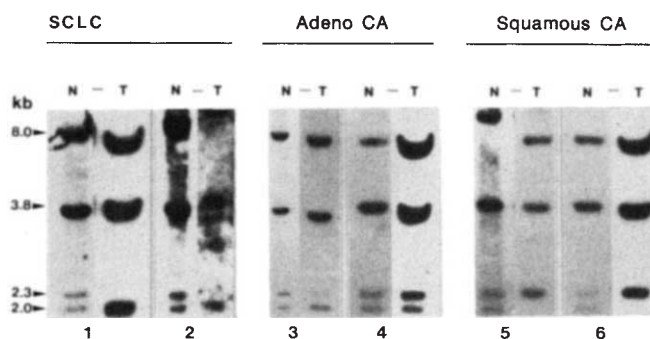


Fig. 3 Southern analysis of *Hind*III-digested DNA from lung tumours and corresponding normal tissue (leukocytes) with pH3H2 showing loss of constitutive heterozygosity. The chromosome 3-specific bands have been aligned. The minor differences in the distance between the two chromosome 1-specific constant bands resulted from the use of different agarose gels. The following tumours are shown: in combination 1, T86-4829 (SCLC); in combination 2, T86-575 (SCLC); in combination 3, T85-9309 (adenocarcinoma of the lung); in combination 4, T86-5602 (adenocarcinoma of the lung); in combination 5, T85-11956 (squamous carcinoma of the lung); in combination 6, T86-6505 (squamous carcinoma of the lung).

Methods. Tumour samples stored at -80°C were minced and suspended in 5 ml STE (100 mM NaCl, 10 mM Tris.HCl, 1 mM EDTA, pH 7.5). Leukocytes, isolated from 40 ml blood, were suspended in 40 ml STE. SDS and proteinase K were added (final concentrations 0.5% w/v and $50\ \mu\text{g ml}^{-1}$ respectively), and the mixture was incubated for 18 h at 37°C . The DNA was purified by phenol/chloroform extractions, ethanol-precipitated, and dissolved in 10 mM Tris.HCl pH 7.5, 0.1 mM EDTA. DNA ($\sim 5\ \mu\text{g}$) was digested to completion with *Hind*III (B.R.L.) and the fragments fractionated by agarose gel electrophoresis and transferred to nylon membrane (Gene Screen Plus). Southern analysis was carried out in the presence of 7% (w/v) SDS³⁰. The probe was labelled by nick translation to a specific activity of $\sim 10^8\ \text{c.p.m.}\ \mu\text{g}^{-1}$.

known heterozygote frequency of 49% (ref. 17). Thus, whereas loss of heterozygosity at 3p21 was evident, no indication of a loss of heterozygosity was found in the same material with this chromosome 13-specific probe (results not shown). In a few cases which showed loss of heterozygosity with pH3H2 we carried out a Southern analysis with another 3p probe, p12-32 (D3S2) (ref. 18). Again a loss of heterozygosity was observed: two SCLC tumours appeared with a homozygous pattern, whereas a squamous cancer remained heterozygous (results not shown). This also confirms that the 3p deletion may include partly different regions of the short arm (see Fig. 1).

Taking medullary carcinoma of the thyroid as a different type of cancer, we found in six tumours a heterozygous pattern with equal band intensities for the 2.3-kb and 2.0-kb *Hind*III fragments detected by pH3H2. Four of them were subjected to densitometry and gave intensity ratios of 1.0–1.2, comparable to the control range. Thus, in this type of tumour we found no indication of allele loss at 3p21. This is not to say that 3p deletions do not occur in other tumours. Clonal abnormalities of chromosome 3 were found in 22 of 50 cases of ovarian adenocarcinoma^{19,20}. The most common abnormality was deletion 3p12, followed by the much more distal deletion 3p25. A deletion distal to 3p22 has also been observed in 5 of 8 cases of anal canal cancer²¹. Although in renal cell carcinoma loss of heterozygosity has been found with pH3H2 (ref. 4 and A.H.v.d.H., K.K., J. W. Oosterhuis, B.C. and C.H.C.M.B., manuscript submitted), 3p12–14 most probably represents the critical region involved in this cancer^{22–24}. Possibly, there is some clustering of epithelial differentiation genes at 3p.

In our present study on lung cancer all informative cases, whatever type of lung cancer they represented, showed loss of heterozygosity at the 3p21 locus DNF15S2 (ref. 25) identified

by pH3H2. This might imply that somatic mutation by loss or inactivation of a gene 3p21 is involved in the development of lung cancer in general, and that by analogy to the situation in cancers with hereditary forms, functional loss of the remaining normal allele is required to unmask the mutant gene.

The results of this study have been presented in part at the Satellite Meeting of the International Congress of Human Genetics on Molecular Genetics and Cytogenetics of Human Neoplasia, Berlin West, 28 September 1986, and at the 9th Meeting of the European Association for Cancer Research, Helsinki, 2 June 1987. We thank Drs W. K. Cavenee, R. White and A. J. Driesel, for making available p9A7, p12-32, and DR2 respectively, and our colleagues Hans Scheffer and Gerard J. te Meerman for helpful discussions. This investigation was supported by the Netherlands Cancer Foundation and the Jan Kornelis de Cock Foundation.

Received 31 July; accepted 5 October 1987.

1. Knudson, A. G. *Cancer Res.* **45**, 1437-1443 (1985).
2. Cavenee, W. K., Koufos, A. & Hansen, M. F. *Mutat. Res.* **168**, 3-14 (1986).
3. Seizinger, B. R., Martuza, R. L. & Gusella, J. F. *Nature* **322**, 644-647 (1985).
4. Zbar, B., Brauch, H., Talmadge, C. & Linchan, M. *Nature* **327**, 721-724 (1987).
5. Solomon, E. *et al.* *Nature* **328**, 616-619 (1987).

6. Whang-Peng, J. *et al.* *Cancer Genet. Cytogenet.* **6**, 119-134 (1982).
7. De Leij, L. *et al.* *Cancer Res.* **45**, 6024-6033 (1985).
8. Wurster-Hill, D. H. *et al.* *Cancer Genet. Cytogenet.* **13**, 303-330 (1984).
9. Zech, L., Bergh, J. & Nilsson, K. *Cancer Genet. Cytogenet.* **15**, 335-347 (1985).
10. Morstijn, G. *et al.* *Cancer Res.* **45**, 3322-3327 (1987).
11. Mooibroek, H., Osinga, J., Postmus, P. E., Carritt, B. & Buys, C. H. C. M. *Cancer Genet. Cytogenet.* **27**, 361-366 (1987).
12. Donlon, T. A. & Magenis, R. E. *HGM7 Cytogenet. Cell Genet.* **37**, 454 (1984).
13. Carritt, B., Welch, H. M. & Parry-Jones, N. J. *Am. J. hum. Genet.* **38**, 428-436 (1986).
14. Goode, M. E., van Tuinen, P., Ledbetter, D. H. & Daiger, S. P. *Am. J. hum. Genet.* **38**, 437-446 (1986).
15. Buys, C. H. C. M. *et al.* *Eur. J. resp. Dis., Suppl.* **70**, 29-36 (1987).
16. Driesel, A. J. *et al.* *HGM8 Cytogenet. Cell Genet.* **40**, 620 (1985).
17. Cavenee, W. K., Leach, P. J., Mohandas, T., Pearson, P. L. & White, R. *Am. J. hum. Genet.* **36**, 10-24 (1984).
18. Naylor, S. L., Johnson, B., Minna, J. D. & Sakaguchi, A. *7th Int. Cong. hum. Genet. Abstr.* **560** (1986).
19. Whang-Peng, J. *et al.* *Cancer Genet. Cytogenet.* **11**, 91-106 (1984).
20. Panani, A. & Ferti-Passantonopoulou, A. *Cancer Genet. Cytogenet.* **16**, 65-71 (1985).
21. Mulieris, M., Salmon, R.-J., Girodet, J., Zafrani, B. & Dutrillaux, B. *Int. J. Cancer* **39**, 595-598 (1987).
22. Pathak, S., Strong, L. C., Ferrell, R. E. & Trinidad, A. *Science* **217**, 939-941 (1982).
23. Wang, N. & Perkins, K. L. *Cancer Genet. Cytogenet.* **11**, 497-481 (1984).
24. Carroll, P. R. *et al.* *Cancer Genet. Cytogenet.* **26**, 253-259 (1987).
25. Willard, H. F., Skolnick, M. H., Pearson, P. L. & Mandel, J.-L. *HGM8 Cytogenet. Cell Genet.* **40**, 360-489 (1985).
26. Ramaekers, F. *et al.* *Histochem. J.* **15**, 691-713 (1983).
27. De Leij, L. *et al.* *Cancer Res.* **45**, 2192-2200 (1985).
28. Buys, C. H. C. M., Koerts, T. & van der Veen, A. Y. *Hum. Genet.* **66**, 361-364 (1984).
29. Harper, M. E. & Saunders, G. F. *Chromosoma* **83**, 431-439 (1981).
30. Church, G. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1991-1995 (1984).

Induction by *E1A* oncogene expression of cellular susceptibility to lysis by TNF

Mann-Jy Chen*, Beverly Holskin*, James Strickler*,
Joselina Gorniak*, Mike A. Clark†, Paul J. Johnson‡,
Michelle Mitcho‡ & David Shalloway‡

* Department of Molecular Genetics and † Department of Molecular Pharmacology, Smith Kline and French Laboratories, King of Prussia, Pennsylvania 19406, USA

‡ Department of Molecular and Cell Biology, The Pennsylvania State University Park, Pennsylvania 16802, USA

Tumour necrosis factor α (ref. 1), synthesized primarily by monocytes in response to various invasive agents, induces a wide variety of biological effects relevant to regulating cell growth and differentiation²⁻⁶, including the selective killing of some tumour cells and the growth stimulation of some normal fibroblasts³. As tumour necrosis factor (TNF) appears to kill tumour cells preferentially, we asked whether TNF sensitivity correlates with the expression of specific oncogene(s). If so, by examining the cellular target(s) of the oncogene product, it might be possible to identify specific factor(s) which mediate TNF action. By using an *in vitro* cytotoxicity assay with NIH 3T3 and Fisher BRK-derived cells expressing exogenously introduced oncogenes, we found that adenovirus *E1A* proteins induce susceptibility to TNF killing.

Cell lines were cloned from foci isolated after transfecting NIH 3T3 cells with plasmids expressing *v-src* (pMvsrc) (ref. 7), human bladder carcinoma activated *ras* (T24-6) or human c-Ha-ras (*ras* C1-2) (S. Yokoyama, personal communication), polyoma middle T or large T antigens (pPyMT1, pLT214) (ref. 8), adenovirus Ad2/Ad5 hybrid (pXC5) (ref. 9) or Ad12 (pAD12E1A) (R. Ricciardi, personal communication) strain E1A, mouse *c-myc* (pZM5) or *v-myc* (pAVM3) (ref. 10) and chicken *c-src* (pMcsrc) (ref. 7), either alone or in pairs. Additional lines expressing *c-myc* were also tested¹¹. The parental NIH 3T3 cells and all its oncogene-transformed derivatives showed little response to recombinant TNF- α , except for NIH(pMcsrc/pXC5) cells which coexpress *c-src* and *E1A* (Fig. 1 and Table 1). NIH(pMcsrc/pXC5)A cells were as sensitive to TNF as standard target L929 cells in a TNF cytotoxicity assay (Fig. 1) (ref. 12), but they were killed more rapidly (within 16 hours) than L929 (died over 2.5 days) (data not shown). Most

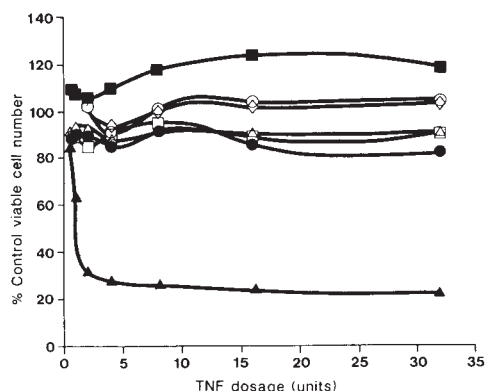


Fig. 1 Response of NIH 3T3 and its oncogene-transformed cell lines to recombinant TNF- α by *in vitro* cytotoxicity assay. NIH 3T3 (■—■), NIH(pMvsrc/focus)A (□—□), NIH(pPyMT1)A (●—●), NIH(pMcsrc/pLT214)A (○—○), NIH(pMcsrc/focus)B (△—△), NIH(pMcsrc/pXC5)A (▲—▲), NIH(T24-6) (◇—◇). All lines were biologically cloned from foci induced by transfection or cotransfection of NIH 3T3 cells with the plasmid identified in parentheses as previously described^{7,10}. To obtain pure bioactive normal human TNF, we cloned the human TNF- α cDNA by constructing and screening a cDNA library of normal human peripheral blood monocytes activated *in vitro* by bacterial endotoxin (data not shown). The library was screened with a synthetic human TNF- α specific probe derived from the published sequence of a cDNA clone from HL-60 human promyelocytic leukaemic cell line¹. Our normal TNF cDNA clone, identical in sequence to the published clones from tumour cells¹, was inserted into an *E. coli* expression vector to produce the mature form of human TNF- α (ref. 1) (data not shown). When purified to homogeneity (as judged by SDS-PAGE, data not shown), the expressed recombinant TNF- α (rTNF- α) usually had a specific activity of $2-4 \times 10^7$ units mg^{-1} protein in our *in vitro* cytotoxicity assay¹ with no added metabolic blocker. Our *in vitro* cytotoxicity assays were modified²⁴ as follows: $1-2 \times 10^4$ L929 cells in 0.1 ml of medium were plated into each well of a 96-well dish. After 12-18 h of incubation at 37 °C in a 5% CO_2 incubator, duplicate twofold serial dilution samples of rTNF- α in 0.1 ml of medium were added to the wells, and the incubation continued for another 48 h. Medium was then removed and the cells washed with phosphate buffered saline, stained with crystal violet and the absorbance at 570 nm measured with a microelisa autoreader (Dynatech)²⁴. The average absorbance of the control wells (not treated with TNF) was taken as 100% viability, and wells treated with 4 M guanidine hydrochloride as 0%. One unit of TNF was defined as the amount of protein needed to kill 50% of L929 cells in a similar assay described above.

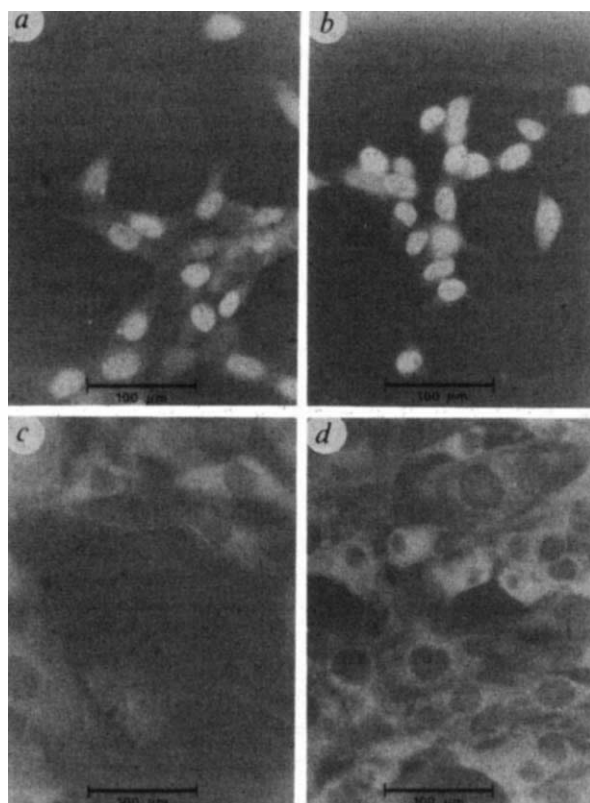


Fig. 2 Expression of *E1A* in transfected NIH 3T3 cell lines assayed by indirect immunofluorescence. Each of the four panels shows the immunofluorescent staining of one cell type: NIH-(pSV2neo/pXC5/mc)A (panel a), NIH(pSV2neo/pXC5/mc)B (panel b), NIH(pSV2neo)D (panel c) and NIH 3T3 (panel d). Cells plated and attached to the coverslips were sequentially washed with phosphate buffered saline (PBS), fixed with 3% formaldehyde in PBS at room temperature for at least 20 min, washed with PBS, made permeable with 0.05% Triton-X100 for 3 min, washed with PBS, then incubated with 20 μ l of 1:1,000 dilution of polyclonal rabbit antiserum raised against recombinant *E1A* (ref. 25) or with 20 μ g ml⁻¹ of monoclonal anti-*E1A* IgG (Clone M73, Ed Harlow, from Oncogene Science, Mineola), at 37 °C for 1 h in 5% CO₂, washed with PBS, incubated with 20 μ l of 1:500 dilution of fluorescein-conjugated IgG goat-anti rabbit immunoglobulins or fluorescein-conjugated IgG goat anti-mouse immunoglobulins (Cappel, Cooper Biomedical, Malvern) as appropriate, rinsed with PBS, mounted on slides and examined by fluorescence microscopy.

NIH(pMcsrc/pXC5) cells expressed *E1A*, as assayed by indirect immunofluorescence technique (Fig. 2 legend, data not shown), and *c-src*, as assayed by immunoprecipitation¹⁰. A residual 20% of the NIH(pMcsrc/pXC5)A cells were resistant to rTNF- α killing; these cells, NIH(pMcsrc/pXC5)A-R, did not express *E1A* (data not shown). Cells from multiple foci induced by cotransfection with *c-src* and either strain of *E1A* were reproducibly sensitized to TNF.

As pMcsrc alone did not induce TNF sensitivity, cells expressing *E1A* but not *c-src* were isolated and tested. We co-transfected NIH 3T3 with pXC5 and pSV2neo, a plasmid which confers G418 resistance¹³, and isolated either individual G418-resistant colonies or mass cultures containing cells pooled from about 50 G418-resistant colonies. *E1A* expression in two independent colony-selected lines and two independent mass cultures was demonstrated by intense nuclear immunofluorescence (Fig. 2, and data not shown). These *E1A* expressing cells were all sensitive to rTNF- α , whereas control clones NIH(pSV2neo)C and D were not (Table 1). Therefore, expression of *E1A* alone is capable of inducing sensitivity to TNF in NIH 3T3 cells.

Both the 289 amino-acid (aa) protein encoded by the *E1A*

Table 1 TNF cytolytic sensitivity of NIH 3T3-derived cell lines and correlation with *E1A* expression

Cell lines*	Genes expressed	Sensitivity†
NIH 3T3 (D.S.)	—	—
NIH 3T3 (R.S.)	—	—
NIH(pMvsrsc/focus)A	<i>v-src</i>	—
NIH(pyMT1)A	polyoma middle <i>T</i>	—
NIH(pMcsrc/pLT214)A	polyoma large <i>T</i>	—
	amino 37K	—
NIH(pMcsrc/focus)B	<i>c-src</i>	—
NIH(pMcsrc/pXC5)A	<i>c-src/E1A</i>	+
NIH(pMcsrc/pAVM3)A	<i>c-src/v-myc</i>	—
NIH(pMcsrc/pAVM3)B	<i>c-src/v-myc</i>	—
NIH(pMcsrc/pAVM3)C	<i>c-src/v-myc</i>	—
NIH(pMcsrc/pZM5)A	<i>c-src/c-myc</i>	—
NIH(pMcsrc/pZM5)B	<i>c-src/c-myc</i>	—
NIH(pMcsrc/pZM5)C	<i>c-src/c-myc</i>	—
NIH(pSVmyc)	<i>c-myc</i>	—
NIH(mycXH)	<i>c-myc</i>	—
NIH(T24-6)	T24 <i>ras</i>	—
NIH(Ras C1-2)	human <i>c-Ha-ras</i>	—
	<i>E1A</i> expression‡	
NIH(pMcsrc/pXC5)A	+	+
NIH(pMcsrc/pXC5)A-R§	—	—
NIH(pMcsrc/pXC5)B	+	+
NIH(pMcsrc/pXC5)C	+	+
NIH(pMcsrc/pXC5)D	+	+
NIH(pMcsrc/pAD12E1A)C	+	+
NIH(pXC5/pSV2neo/mc)A	+	+
NIH(pXC5/pSV2neo/mc)B	+	+
NIH(pXC5/pSV2neo/cos)A	+	+
NIH(pXC5/pSV2neo/cos)B	+	+
NIH(pSVneo)C	—	—
NIH(pSV2neo)D	—	—

* NIH 3T3 cells from two different sources were tested. NIH(pSVmyc) and NIH(mycXH) are previously characterized NIH 3T3-derived cell lines which express *c-myc* (ref. 11). NIH(pXC5/pSV2neo/mc)A and B are independent mass cultures of G418-resistant cell populations which were isolated after cotransfections with pXC5 and pSV2neo (ref. 10). NIH(pXC5/pSV2neo/cos)A and B are two clonal cell lines selected with G418 after cotransfection with pXC5 and pSV2neo. NIH(pSV2neo)C and D are two control cell lines selected with G418 after transfection with pSV2neo alone. All other lines were cloned from foci induced by transfection or cotransfection of NIH 3T3 cells with the plasmids identified within parentheses^{7,10}.

† Sensitivity was assayed by *in vitro* cytotoxicity assay, as described in the legend to Fig. 1.

‡ *E1A* expression was tested by indirect immunofluorescence staining as described in the legend to Fig. 2. pXC5 expresses an Ad2/Ad5 hybrid *E1A* and pAD12E1A expresses Ad12 *E1A*.

§ NIH(pMcsrc/pXC5)A-R is a TNF-resistant cell population derived after repeated treatment of NIH(pMcsrc/pXC5)A with TNF.

13S messenger RNA and the 243aa protein encoded by the 12S mRNA are expressed from pXC5. The 243aa *E1A* lacks a domain apparently important in activating the transcription of other adenoviral genes¹⁴. To determine if this function is required for induction of TNF sensitivity, we tested NIH 3T3 cells and Fisher BRK cells expressing these *E1A* proteins separately. Table 2 shows that the expression of either protein, whether from complementary DNA (p13Swt, p12Swt) (ref. 15) or mutated genomic clone fragments (pEKpm975, pEKdl1500 (ref. 16), can sensitize NIH 3T3 cells to TNF. All of the *E1A*-expressing lines were as sensitive to TNF as NIH(pMcsrc/pXC5) lines, except that four of the six lines expressing 12S *E1A* mRNA alone were 5–25-fold less sensitive (data not shown). These results indicate that the unique domain present in the 13S and absent in the 12S *E1A* mRNA is helpful, but not essential, for the induction of TNF sensitivity, and that the induction of cellular sensitivity to TNF by *E1A* is not limited to NIH 3T3 cells, because Fisher BRK cells expressing *E1A* were also sensitized to TNF (Table 2, group D).

TNF may be important in tumour cell destruction by activated macrophages¹⁷ and natural cytotoxic cells¹⁸. It has been recently reported¹⁹ that both the 289aa and 243aa Ad5 *E1A* proteins can induce susceptibility of rodent cells to lysis by host inflammatory cells, natural killer (NK) cells and activated macrophages. These results strongly suggest that TNF is the major cytolytic factor

responsible for the killing of *E1A*-transformed cells by activated macrophages. This hypothesis is further supported by our preliminary data (in collaboration with J. Cook) indicating that NIH 3T3 cells expressing *E1A* are also sensitive to activated rat macrophages. Both activated macrophages and NK cells could provide an initial barrier to neoplastic cell proliferation *in vivo* during the induction of specific cellular immunity²⁰. Whereas both Ad2- and the highly oncogenic Ad12-transformed rat cells are highly susceptible to killing by BCG-activated macrophages, only Ad2- but not Ad12-transformed hamster cells are relatively susceptible to NK cells¹⁷. In our system, however, both the Ad2/Ad5 hybrid and the Ad12 *E1A* induced TNF sensitivity. Therefore, it is likely that factor(s) other than TNF are involved in the cytolysis of adenovirus-transformed cells by NK cells²¹.

It seems that immortalization by *E1A* is not directly relevant to the induction of sensitivity to TNF as two other immortalizing oncogenes, *myc* and polyoma large *T*, do not induce TNF sensitivity. Moreover, the parental NIH 3T3 cells are already immortalized. However, though transcriptional transactivation of some cellular genes by *E1A* may be involved. The fact that the 12S product is a less effective transactivator than the 13S product¹⁴ could account for its reduced ability to induce TNF sensitivity. Because both *E1A* proteins can suppress the transcription of some cellular genes (such as fibronectin²²), it is also possible that gene suppression by *E1A* could cause the induction of TNF sensitivity.

Testing the sensitivity of cells expressing mutant *E1A* proteins may reveal the domain(s) and thereby the function(s) of *E1A* that are required to induce TNF sensitivity. Recent study suggests the presence of a specific TNF receptor peptide on TNF-

cytolysis susceptible cells²³. *E1A* expression could induce the appearance of such a TNF receptor peptide.

We thank Sita Reddy for cell line NIH(pPyMT1)A, M. D. Cole for cell lines NIH(pSVmyc) and NIH(mycXH), J. Cook for cell lines Fisher BRK (MT1A) and Fisher BRK (12S), S. Yokoyama and R. Sweet for cell lines NIH(T24-6) and NIH(ras Cl-2), and A. Berk (pEKdl1500, pEKpm975), R. Kamen (pLT214, pPyMT1), B. Moran (pE1AwT, p12Swt, p13Swt), L. Parada and R. Weinberg (pAVM3, pZM5), R. Ricciardi (pAD12E1A), and D. Spector (pXC5) for plasmids. We also thank Martin Rosenberg and Raymond W. Sweet for critical reading of the manuscript. This work was supported in part by PHS and an American Cancer Society Junior Faculty Research Award (to D.S.).

Received 3 August; accepted 19 October 1987.

1. Pennica, D. *et al.* *Nature* **312**, 724-729 (1984).
2. Beutler, B. & Cerami, A. *Nature* **320**, 584-588 (1986).
3. Sugarman, B. J. *et al.* *Science* **230**, 943-945 (1985).
4. Munker, R., Gasson, J., Ogawa, M. & Koefler, H. P. *Nature* **323**, 79-82 (1986).
5. Takeda, K. *et al.* *Nature* **323**, 338-340 (1985).
6. Pekala, P. H., Kawakami, M., Angus, W., Lane, M. D. & Cerami, A. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2743-2747 (1983).
7. Johnson, P. J., Coussens, P., Denko, A. & Shalloway, D. *Molec. cell. Biol.* **5**, 1073-1083 (1985).
8. Rassoulzadegan, N. *et al.* *Nature* **300**, 713-718 (1982).
9. Spector, D. J. & Tevethia, M. J. *Virology* **151**, 329-338 (1986).
10. Shalloway, D., Johnson, P. J., Freed, E. O., Coulter, D. & Flood, W. A. Jr *Molec. cell. Biol.* **7**, 3582-3590 (1987).
11. Keith, E. J., Caimi, P. G. & Cole, M. D. *Cell* **39**, 339-348 (1984).
12. Ruff, M. R. & Gifford, G. E. in *Lymphokines* Vol. 2 (ed. Pick, E.) 235-272 (Academic, New York, 1981).
13. Southern, P. J. & Berg, P. J. *Molec. appl. Genet.* **1**, 327-341 (1982).
14. Berk, A. J. *A. Rev. Genet.* **20**, 45-79 (1986).
15. Zerler, B. *et al.* *Molec. cell. Biol.* **6**, 887-899 (1986).
16. Montell, C., Courtois, G., Eng, C. & Berk, A. *Cell* **36**, 951-961 (1984).
17. Urban, J. L., Shepard, H. M., Rothstein, J. L., Sugarman, B. J. & Schreiber, H. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5233-5237 (1986).
18. Ortolano, J. R. *et al.* *Nature* **321**, 700-702 (1986).
19. Cook, J. L. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **83**, 6965-6969 (1986).
20. Lewis, A. M. Jr & Cook, J. L. *Science* **227**, 15-20 (1985).
21. Herberman, R. (ed.) *Mechanisms of Cytotoxicity by NK-cells* (Academic, New York, 1985).
22. Roberts, B. E. *et al.* *J. Virol.* **56**, 404-413 (1985).
23. Creasey, A. A., Yamamoto, R. & Vitt, C. R. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3293-3297 (1987).
24. Aggarwal, B. B., Moffat, B. & Harkins, R. N. *J. biol. Chem.* **259**, 686-691 (1984).
25. Ferguson, B., Krippl, B., Andrisani, O., Westphal, H. & Rosenberg, M. *Molec. cell. Biol.* **5**, 2653-2661 (1985).

Table 2 Induction of TNF cytolytic sensitivity by *E1A* protein from 12S or 13S mRNA

Cell lines	<i>E1A</i> expression	Sensitivity
Group A (13S + 12S):		
NIH(pE1AwT/pSV2neo/cos)A	+	+
Group B (13S only):		
NIH(p13Swt/pSV2neo/cos)A	+	+
NIH(p13Swt/pSV2neo/cos)B	+	+
NIH(p13Swt/pSV2neo/cos)C	+	+
NIH(pEKpm975/pSV2neo/cos)A	+	+
NIH(pEKpm975/pSV2neo/cos)C	+	+
NIH(pEKpm975/pMscrc)A	+	+
NIH(pEKpm975/pMscrc)D	+	+
Group C (12S only):		
NIH(p12Swt/pSV2neo/cos)E	+	+
NIH(p12Swt/pSV2neo/cos)F	+	+
NIH(p12Swt/pSV2neo/cos)G	+	+
NIH(p12Swt/pSV2neo/cos)H	+	+
NIH(p12Swt/pSV2neo/cos)I	+	+
NIH(p12Swt/pMscrc/pSV2neo/cos)E	+	+
NIH(p12Swt/pMscrc/pSV2neo/cos)F	+	+
NIH(p12Swt/pMscrc/pSV2neo/cos)G	+	+
NIH(p12Swt/pMscrc/pSV2neo/cos)H	+	+
NIH(p12Swt/pMscrc/pSV2neo/cos)I	+	+
NIH(p12Swt/pMscrc/pSV2neo/cos)J	+	+
NIH(pEKdl1500/pMscrc/pSV2neo/cos)C	+	+
NIH(pEKdl1500/pMscrc/pSV2neo/cos)D	+	+
Group D (other cell types):		
Fisher BRK (MT1A)	+	+
Fisher BRK (12S)	+	+

Both the 12S and the 13S *E1A* proteins are capable of inducing TNF sensitivity. *In vitro* cytotoxicity assays and indirect immunofluorescence staining for *E1A* expression are described in the legends to Figs 1 and 2 respectively. Expression plasmid pE1AwT expresses both the 12S and the 13S mRNA *E1A* protein. Expression plasmid p13Swt and p12Swt were constructed with complementary DNA clones of the 13S or the 12S *E1A* mRNA (ref. 15). Expression plasmids pEKpm975 and pEKdl1500 were constructed with *E1A* genomic clones expressing only the 13S or the 12S translated *E1A* because of a mutation at a 12S mRNA splice junction, and a small deletion at a 13S mRNA splice junction respectively¹⁶. Cell lines were isolated after cotransfection of NIH 3T3 cells with the plasmids indicated in parentheses, as described¹⁰. Cell lines which had been cotransfected with pSV2neo were clonally selected with G418; other lines were cloned from foci. Fisher BRK (MT1A) cells express both the 13S and the 12S *E1A* mRNA products and Fisher BRK (12S) cells express only the 12S mRNA product¹⁹.

Maternal regulation of *zerknüllt*: a homoeobox gene controlling differentiation of dorsal tissues in *Drosophila*

Christine Rushlow, Manfred Frasch, Helen Doyle & Michael Levine

Department of Biological Sciences, Fairchild Center, Columbia University, New York, New York 10027, USA

The homoeobox gene *zerknüllt* (*zen*) plays an important role in the differentiation of dorsal tissues during *Drosophila* development^{1,2}. *zen*⁻ embryos show transformations in the dorsal-most regions of the fate map, and lack several tissues that normally derive from these regions, including the amnioserosa and optic lobe¹. *zen* displays a simple dorsal on/ventral off pattern as early as cleavage cycle 10-11 (ref. 2). We have prepared a polyclonal antibody against a full-length *zen* protein, and used this to examine its pattern of expression in mutants that disrupt dorsal-ventral polarity. Most or all of the maternally expressed genes that are involved in this process have been previously identified and fall into two classes, so called 'dorsalizers' and 'ventralizers' (see refs 4-7, reviewed in ref. 8). On the basis of our analysis of *zen* expression in each of these maternal mutants we propose that one or more of the dorsalizing genes encodes a repressor which inhibits the expression of *zen* in ventral regions of developing embryos. The ventralizing gene *cactus*⁷ might play an important role in

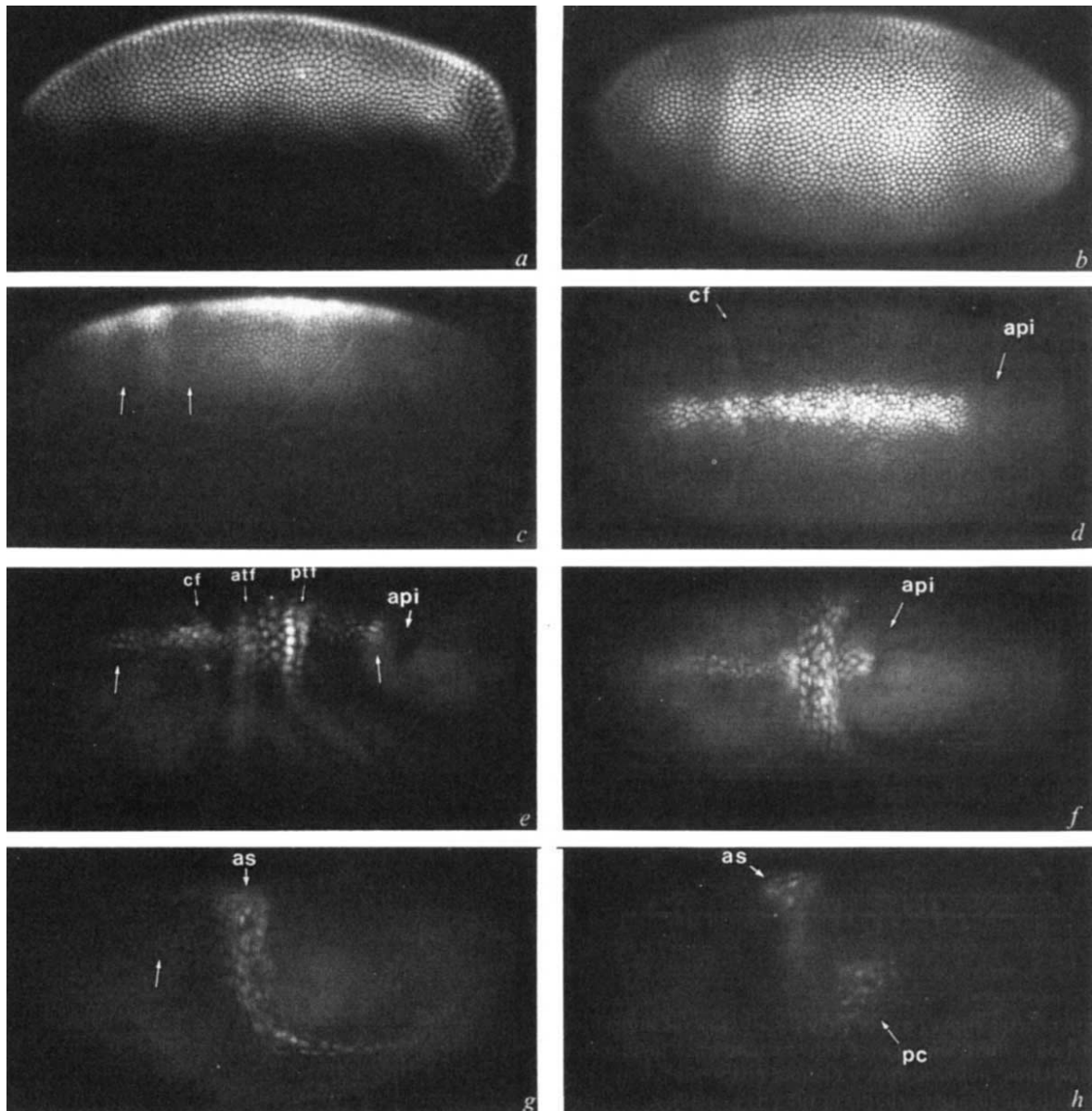


Fig. 1 Immunolocalization of the *zen* protein in wild-type embryos. Whole mount preparations of fixed wild-type embryos were stained with affinity purified anti-*zen* antibodies, and visualized by indirect immunofluorescence using rhodamine-conjugated goat anti-rabbit antibodies. All embryos in this and subsequent figures are oriented so that anterior is to the left; lateral views are oriented so that dorsal is up. *a-g*, Lateral views of progressively older wild-type embryos, and *b-f*, dorsal views of similar stages. *a*, and *b*, Cleavage stage 14 embryos just prior to the completion of cellularization. Nuclei located in dorsal regions (about 40% of the embryo's circumference) and at the poles are stained by the antibody. There is no staining above background levels in ventral regions. *c*, Lateral view of cellularized embryo. Note that the staining along the dorsal surface is more intense than that seen in more lateral regions. Staining is no longer detected at the anterior and posterior poles. Also, the staining seen on the dorsal surface is discontinuous, with the regions at ~77 to 71% and ~65 to 61% egg length showing lower levels of staining (arrows). *d*, Dorsal view of a gastrulating embryo. By this time the staining has become restricted to the dorsal-most ectoderm, in a strip about 5-6 cells wide. *e* and *f*, Embryos undergoing germ band elongation. During this time much of the dorsal staining corresponds to the differentiating amnioserosa. But, staining is also seen in regions just anterior and posterior to the limits of the amnioserosa (arrows), including the optic lobe primordia, as well as the posterior-most portion of the germ band. *g* and *h*, An embryo that has completed germ band elongation is shown in two different planes of focus. Strong staining is seen in each of the cells that comprise the amnioserosa. A weaker patch of staining is observed in the procephalon (arrow), which probably corresponds to the presumptive optic lobe. Strong staining is also detected in a subset of the pole cells within the PMG. Abbreviations: api, amnioproctodeal invagination; as, amnioserosa; atf, anterior transverse furrow; cf, cephalic furrow; pc, pole cells; ptf, posterior transverse furrow.

Methods. A *zen* complementary DNA that contains the entire protein coding sequence⁹ was cloned into the T7 expression vector, pAR3040¹². BL21(DE3) bacterial cells containing the T7/*zen* recombinant were induced to express *zen* protein by the addition of isopropyl thiogalactoside (IPTG) as described by Studier and Moffatt¹². Enriched fractions of the *zen* protein were prepared for immunizing New Zealand White rabbits as described by Frasch *et al.*¹³. The preparation of affinity purified anti-*zen* antibodies and the staining of whole mount preparations of embryos were also done exactly as described by Frasch *et al.*¹³.

restricting the activity of this repressor to ventral regions, thereby permitting the activation of *zen* in those dorsal tissues where its function is critically required.

The *zen* gene was originally cloned on the basis of homoeobox cross-homology using the homoeobox sequence derived from the homoeotic gene, *Sex combs reduced* (*Scr*)². More recently we have found that there are two closely linked homoeobox transcription units within the *zen* region of the *Antennapedia* gene complex, called *z1* and *z2* (ref. 9). These genes show essentially identical patterns of expression; however, several lines of evidence suggest that *z1* corresponds to *zen* and *z2* might be dispensable⁹. A full-length *z1* (hereafter called *zen*) protein was prepared in *Escherichia coli* and used to prepare rabbit antiserum that recognizes the native protein in *Drosophila*. The distribution of this protein during wild-type development closely parallels the timing and sites of *zen*⁺ activity based on previous genetic studies¹.

The *zen* protein is first detected in wild-type embryos undergoing the 13th nuclear cleavage, about two hours after fertilization. Staining is broadly distributed over the dorsal surface of the embryo (and encompasses about 40% of the embryo's circumference), as well as at the anterior and posterior poles (Fig. 1a, b). At the onset of cleavage cycle 14, *zen* staining is essentially uniform along the anterior-posterior axis, but within the next ~10 min reduced levels are observed at ~77 to 71% and ~65 to 61% egg length (arrows, Fig. 1c). There is a refinement of the *zen* pattern during cell cycle 14 development, such that the *zen* protein becomes progressively more restricted to the dorsal-most regions of the embryo. Also, staining is no longer observed at the anterior and posterior poles. When gastrulation begins, the *zen* protein is localized to a dorsal strip ~5–6 cells wide (~10% of the embryo's circumference) that extends from ~85 to ~28% egg length (Fig. 1c, d).

Staining of the dorsal-most ectoderm persists during germ band elongation, and following the completion of this process *zen* expression is not detected for the remainder of the life cycle. Most of the cells that are stained by the antibody correspond to the differentiating amnioserosa (Fig. 1e–g). The *zen* protein is also detected in tissues adjacent to the amnioserosa, including the optic lobe primordia¹⁰ (Fig. 1e, f, arrows). Both the amnioserosa and optic lobe are absent in advanced-stage *zen*[–] embryos¹. Transient expression is also observed in a subpopulation of the pole cells contained within the posterior midgut (PMG) (Fig. 3h), although there is no known role for *zen*⁺ gene activity in these cells.

In an effort to identify possible regulatory interactions involved in the establishment and maintenance of the *zen* expression pattern, we have analysed the distribution of the *zen* protein in each of the 12 known maternal-effect mutants that disrupt dorsal-ventral polarity^{4–8}. Each of the 11 dorsalizing mutants displays an essentially identical alteration of the *zen* protein pattern, whereby expression is observed in both dorsal and ventral regions (Fig. 2). An abnormal *zen* pattern is detected at the earliest time when staining above background levels is first observed (during the 13th nuclear cleavage), and all of the embryonic nuclei display uniformly intense staining (Fig. 2a). Tissue hybridization studies reveal mis-expression of *zen* RNAs at substantially earlier stages, by cleavage cycle 10–11 (data not shown). An abnormal *zen* pattern persists in mutants undergoing cellularization and gastrulation (Fig. 2b, c), however, during this time substantially lower levels of staining are detected at the anterior and posterior poles. Additional heterogeneities become apparent during gastrulation, such that the *zen* staining pattern is divided into three broad 'bands', including two bands near the anterior pole (between ~85 and 65% egg length; see Fig. 2c). A similar discontinuous *zen* pattern is also observed along the dorsal surface of wild-type embryos (see Fig. 1c), but is more pronounced in the mutants as *zen* staining includes both dorsal and ventral regions.

The *zen* staining pattern observed in *Toll*[–] embryos is essen-

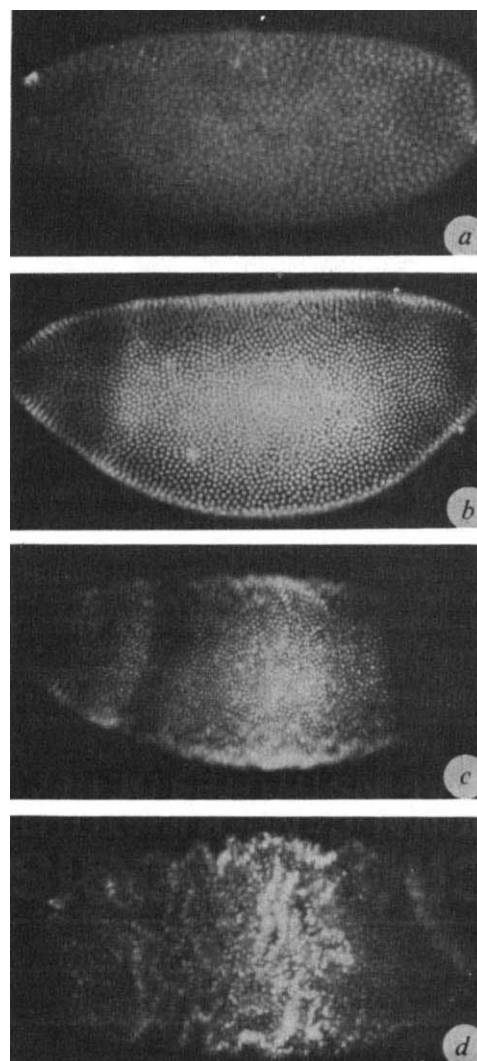


Fig. 2 The *zen* staining pattern in a 'dorsalized' maternal mutant. Embryos were collected from *pipe* homozygous females and stained with affinity purified anti-*zen* antibodies. *a*, Pre-cellular embryo undergoing the 13th nuclear cleavage. There is essentially uniform staining of all nuclei, in both dorsal and ventral regions. A similar pattern of mis-expression of the *zen* pattern is also seen in each of the other 11 dorsalizing maternal mutants (data not shown). *b*, Cycle 14 embryo undergoing cellularization. Strong staining persists in dorsal and ventral regions. But, discontinuities in the pattern are observed along the anterior-posterior axis, and there is a loss of staining at the anterior and posterior poles. *c*, Gastrulating embryo. The poles are no longer stained, and the discontinuities of the pattern are more pronounced along the anterior-posterior axis. Two broad bands of staining can be seen near the anterior pole (from ~85 to ~65% egg length), while more or less uniform staining is observed in the middle body region, from ~61 to 28% egg length. *d*, Post-gastrulating embryo (~4–4½ h after fertilization). Staining persists in the middle body region. Note the folded appearance of the embryo, which is typical of dorsalizing mutants.

tially identical to that seen in each of the ten other dorsalizing mutants described above. In contrast, dominant gain of function mutations in *Toll* (that is, *Toll*^D mutations) result in a very different pattern of *zen* staining, which is consistent with their 'ventralizing' phenotypes^{5,6}. In such mutants, *zen* staining is not observed in either dorsal or ventral regions, and is detected only at the anterior and posterior poles during pre-cellular stages (Fig. 3a). After cellularization, the only staining that is observed appears to correspond to one or two secondary yolk nuclei located near the posterior pole (Fig. 3b). The *zen* pattern

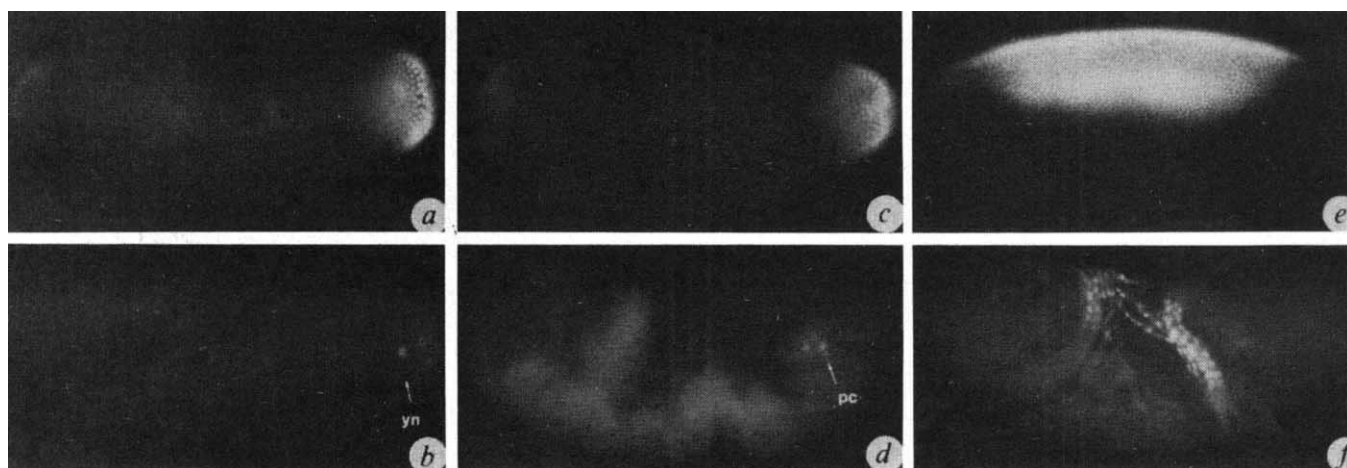


Fig. 3 *zen* staining pattern of *Toll^D*, *cactus* and *torso* mutants. *a* and *b*, Embryos obtained from *Toll^D* females. *c* and *d*, Embryos obtained from *cactus*⁻ females. *e* and *f*, Embryos obtained from *torso*⁻ females. *a*, A pre-cellular *Toll^D* embryo. Staining is not detected above background levels in the middle body region in either dorsal or ventral regions. Only somatic nuclei located at the anterior and posterior poles are stained. *b*, *Toll^D* mutant at the beginning of gastrulation. The polar staining has disappeared, and the only staining that is observed (arrow) corresponds to one or two secondary yolk nuclei located near the posterior pole. *c*, *cactus*⁻ Embryo just prior to the completion of cellularization. The staining pattern is very similar to that observed in *Toll^D* mutants. Normal levels of staining are observed at the anterior and posterior poles; however, there is no expression detected above background levels in the middle body region. *d*, *cactus*⁻ Embryo after gastrulation, ~4-4½ h post-fertilization. Several of the pole cells are stained, but there is no expression in other regions of the embryo. Note the abnormal location of the PMG, and the large cleft on the ventral surface. This mutant phenotype is similar to *zen*⁻ embryos of similar age. *e*, *torso*⁻ Embryo during cellularization. A normal 'dorsal on/ventral off' *zen* pattern is observed in the middle body region. But, there is no staining above background levels at the poles. *f*, *torso*⁻ Embryo undergoing germ band elongation. Staining is observed in the amnioserosa, and in adjacent regions, as observed in wild type. None of the pole cells within the PMG are stained. Abbreviations as for Fig. 2.

observed in the ventralizing mutant *cactus*⁷ is virtually identical to that seen in *Toll^D* mutants (Fig. 3*c, d*), although unlike *Toll^D*, *cactus*⁻ embryos show *zen* staining in a subset of the pole cells within the PMG (Fig. 3*d*).

The expression of *zen* at the poles as opposed to the middle body regions appears to involve separate regulatory cues. *zen* transcripts are detected at the poles prior to their expression in middle body regions of wild-type embryos (C.R. and H.D., unpublished). Also, polar expression is transient and only detected during pre-cellular stages, whereas middle body staining persists during gastrulation and germ band elongation (Fig. 1*a* and *c*). Transient expression at the poles appears normal in both dorsalizing and ventralizing mutants, even though these mutants strongly perturb expression in the middle body region (Figs 2*b, c* and 3*a-d*). We have examined the *zen* pattern in maternal mutants that disrupt the differentiation of the anterior-posterior pattern; two of these, *torso* and *trunk*¹¹, eliminate *zen* staining at the poles, but do not disrupt the normal 'dorsal on/ventral off' pattern in the middle region (Fig. 3*e, f*). Both of these mutants cause deletions in the anterior- and posterior-most structures of advanced-stage embryos¹¹.

We have shown that the normal 'dorsal on/ventral off' *zen* pattern is disrupted in mutations for each of the 12 different maternal-effect genes that are required for the establishment of the dorsal-ventral pattern during early development. Previous studies on the role of these genes have relied primarily on the morphology of cuticular structures secreted by the epidermis⁴⁻⁶. As *zen* products participate in the differentiation of dorsal structures (that is, the amnioserosa and optic lobe) that do not contribute to cuticular pattern elements¹, it was not clear from the previous studies whether any of the maternal-effect genes influence the pattern of *zen* expression. Our results suggest that one or more of the 11 dorsal group genes either directly or indirectly encodes a 'repressor' that inhibits the expression of *zen* in ventral regions of wild-type embryos. Thus, mutations in any of the 11 dorsal group genes might disrupt the activity of this repressor, resulting in the observed ectopic expression of *zen*. Previous genetic studies^{5,6}, in combination with the staining

data presented here, are consistent with the hypothesis that *Toll^D* encodes a constitutively active repressor that inhibits *zen* expression in both dorsal and ventral regions (see Fig. 3).

There are at least two explanations for the absence of *zen* products in the middle body region of *cactus* mutants. It is possible that *cactus* directly or indirectly specifies an activator that participates in the initiation of *zen*. Alternatively, *cactus* could modulate the activity of a repressor encoded by the dorsal group genes, restricting active repressor to ventral regions of wild-type embryos. The observation that *Toll^D* and *cactus* mutants display essentially identical patterns of *zen* expression is consistent with this latter 'anti-repressor' mechanism. Thus, *cactus*⁻ mutants might contain active repressor in both dorsal and ventral regions, thereby preventing the activation of *zen*. Further studies will be required to distinguish between an activator or anti-repressor mechanism, including an analysis of the *zen* staining pattern in *cactus*/dorsal-group double mutants, and the expression of truncated *zen* promoters in *cactus* mutants by P-transformation.

We thank Thom Kaufman, Ruth Steward, Kathryn Anderson and Trudi Schupbach for fly stocks. We gratefully acknowledge Christiane Nusslein-Volhard for helpful discussions and for sharing unpublished results. We thank Patrick Lo for help with the staining of *torso* mutants. This work was funded by the NIH and the ACS.

Received 3 August; accepted 20 October 1987.

1. Wakimoto, B. T., Turner, F. R. & Kaufman, T. C. *Devl Biol.* **102**, 147-172 (1984).
2. Doyle, H. J., Harding, K., Hoey, T. & Levine, M. *Nature* **323**, 76-79 (1986).
3. Levine, M. & Harding, K. *Oxford Survey on Eukaryotic Genes* **5**, (in the press).
4. Anderson, K. V. & Nusslein-Volhard, C. *Nature* **311**, 223-227 (1984).
5. Anderson, K. V., Jurgens, G. & Nusslein-Volhard, C. *Cell* **42**, 779-789 (1985).
6. Anderson, K. V., Bokla, L. & Nusslein-Volhard, C. *Cell* **42**, 791-798 (1985).
7. Godt, D. thesis, Univ. München (1984).
8. Anderson, K. V. *Trends genet. Sci.* **3**, 91-97 (1987).
9. Rushlow, C., Doyle, H., Hoey, T. & Levine, M. *Genes Dev.* (in the press).
10. Campos-Ortega, J. & Hartenstein, V. in *The Embryonic Development of Drosophila melanogaster* 31-36 (Springer, Berlin, 1986).
11. Schupbach, T. & Wieschaus, E. *Roux Arch. dev. Biol.* **195**, 302-317 (1986).
12. Studier, F. W. & Moffat, B. A. *J. molec. Biol.* **189**, 113-130 (1986).
13. Frasch, M., Hoey, T., Rushlow, C., Doyle, H. & Levine, M. *EMBO J.* **6**, 749-759 (1987).

Constitutive expression of (2'-5') oligo A synthetase confers resistance to picornavirus infection

J. Chebath, P. Benesh, M. Revel & M. Vigneron*

Department of Virology, Weizmann Institute of Science, Rehovot, Israel

Study of the mechanisms by which interferon (IFN) treatment of cells induces resistance to virus infections has been complicated by the multiple biochemical changes induced. Over 20 proteins are increased by IFN, including the double-stranded (ds) RNA-activated protein kinase, (2'-5') oligo A synthetase, surface proteins such as the major histocompatibility complex (MHC) proteins, and various proteins with unknown functions¹⁻³. The availability of cloned complementary DNAs for several IFN-induced proteins³⁻⁸ now allows us to probe their roles in IFN action. For instance, the murine Mx protein has been shown to confer resistance to influenza virus⁷. We studied chinese hamster ovary (CHO) cell clones expressing high constitutive levels of (2'-5') A synthetase as a result of transfection with the cDNA encoding the enzyme form⁸ which has a relative molecular mass (M_r) of 40K. Elevated enzyme correlates directly with resistance to infection by a picornavirus such as Mengo, but does not make the cells resistant to vesicular stomatitis virus (VSV).

The (2'-5') A synthetase is one of the *in vitro* translation inhibitors found in extracts of IFN-treated cells^{1,2}. It polymerizes ATP into ppp(A2'p)_nA oligomers⁹ which activate a latent ribonuclease^{1,2}. Previously we reported the sequence of two

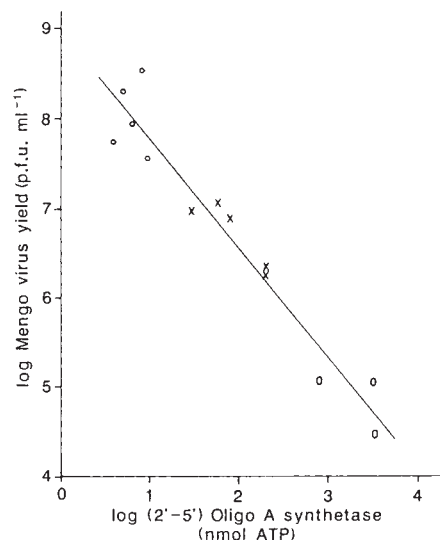


Fig. 2 Reduction of Mengo virus yield in CHO cells with increased levels of (2'-5') oligo A synthetase. The titres of Mengo virus (measured on mouse L cells as described in ref. 24) obtained in CHO cells 24 h after infection at MOI = 1, are indicated for five CHO-DHFR clones (small open circles), five CHO-E16 clones (crosses) and three amplified CHO-E16 clones (large open circles) in relation to the (2'-5') A synthetase activity measured in the same clones by ³²P-ATP incorporation into (A2'p)_nA cores (see ref. 13) and expressed as log pmol ATP incorporated with 2 µg protein in 2 h at 30 °C.

* Present address: LGME, 11 rue Humann, Strasbourg, France.

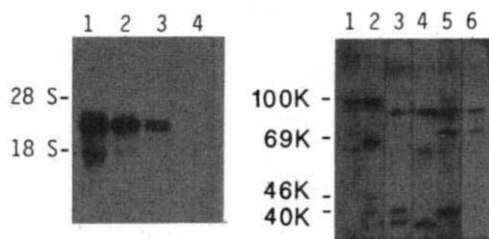


Fig. 1 Constitutive expression of (2'-5') oligo A synthetase. Left, Northern electrophoretic blot of 50 µg RNA from CHO-E16 clones 3-17-12 (lane 1), 3-17-8 (lane 2), 3-17 (lane 3) and CHO-DHFR clone 1-11 (lane 4), hybridized to human (2'-5') A synthetase cDNA 9-21 nick-translated with ³²P-dCTP⁸. The upper band is the expected 2.4-kb transcript ending at the SV40 poly(A) site. Right, Western electrophoretic blot of 30 µg protein from 0.5% Nonidet P-40 extracts of human SV80 cells, untreated (lane 1) or after 18 h with 200 U ml⁻¹ rHuIFN-β₁ (lane 2), or from untreated CHO-E16 clone 3-17-8 (lanes 3, 5), or untreated CHO-DHFR clone 1-8 (lanes 4, 6). CHO proteins were from a 100,000g supernatant (lanes 3, 4) or from the microsomal pellet (lanes 5, 6). After polyacrylamide gel electrophoresis in sodium dodecyl sulphate, proteins were blotted on nitrocellulose and reacted with anti-peptide B antibodies and ¹²⁵I-labelled protein A¹⁰. The position of the four (2'-5') A synthetase induced in human cells by IFN¹⁰ is indicated.

Methods. To construct pE-E16, the E16 cDNA 9-21 (ref. 8) was excised with *EcoRI*, blunted and ligated in *HindIII*-cut blunted pSVE3 DNA¹². The initiator ATG is 97 base-pairs (bp) downstream from the RNA start of SV40 *T-ag* RNA start and the E16 cDNA segment (1.275 kilobases (kb)) is followed by 879 bp from the 3' end of the *T-ag* gene including the polyadenylation site¹². Cotransfection of CHO DHFR⁻ cells by pE-E16 and pSVDHFR, selection of DHFR⁺ clones and amplification by MTX selection, was done as previously described¹².

cDNAs encoding active human (2'-5') A synthetase^{8,10}, one for a 1.6 kilobase RNA (E16) and 40K protein, the other for a 1.8 kb RNA produced by differential splicing and a 46K enzyme with a different C-terminus. Antibodies to a common peptide of the 40 and 46K enzymes recognize two more IFN-induced enzyme forms, a 100K enzyme which is ribosome-bound and does not require dsRNA, and a 69K enzyme which is membrane-bound and, like the two small forms, dependent on dsRNA¹⁰. In this work, cells with high constitutive levels of (2'-5') A synthetase were obtained by cotransfection of CHO DHFR⁻ cells¹¹ with pSVDHFR¹² and the human E16 cDNA clone 9-21 (ref. 8) fused to an SV40 early promoter (pE-E16).

The (2'-5') A synthetase activity¹³ increased from 3.2 ± 0.9 pmol ATP per µg of protein in CHO-DHFR clones (with pSVDHFR only) to 57 ± 35 pmol ATP per µg of protein in a series of CHO-E16 clones transformed by pE-E16 and pSVDHFR. By selection in 100 nM methotrexate (MTX), CHO-E16 clone 3-17 yielded subclones 8, 9 and 12 with amplified transgenomes and enzyme levels of 390, 1,500 and 1,560 respectively. Such 100-500-fold increases over the basal level are equivalent to treating CHO-DHFR cells with 5,000U per ml human IFN-β₁. RNA blots (Fig. 1) showed that the enzyme level in the amplified clones correlates with accumulation of the expected messenger RNA hybridizing to E16 cDNA. No hybridization was seen in CHO-DHFR and no IFN was detected in the medium of clones with high E16 expression. Immunoblots with human specific antibodies showed accumulation of the 40K protein (Fig. 1). No 46K or 69K forms were seen but a 100K protein became apparent in microsomes of CHO-E16 clone 3-17-8, suggesting that the E16 cDNA can also give rise to this large ribosome-bound enzyme¹⁰.

When challenged with Mengo virus infection, the three clones with highest constitutive (2'-5') A synthetase (~2 × 10⁴ molecules per cell) showed a thousandfold decrease in virus yield compared to the CHO-DHFR clones (50 molecules per cell) (Fig. 2). A direct correlation with a linear regression coefficient of -1.2 ± 0.1 was seen between the log (2'-5') A synthetase level and the log Mengo virus titre in the 13 clones

Table 1 Reduction in Mengo virus yield in CHO-E16 clone 3-17-12*

		Multiplicity of infection (p.f.u. per cell) [†]			
	Time (h)	0.01	0.05	1	10
		log p.f.u. per ml			
Expt 1	12	3.32	2.21	—	—
	24	3.16	2.15	2.79	2.4
	36	4.21	2.88	—	—
Expt 2	12	2.07	2.39	2.23	1.45
	24	2.55	2.23	1.6	1.28
	36	3.18	2.84	1.05	2.0

* Compared to control CHO-DHFR clone 1-8. Log of virus p.f.u. ml⁻¹.

† Multiplicity of infection, p.f.u. per cell, for both cell lines.

Table 2 Virus concentration needed to produce cytopathic effect

Clone	Multiplicity of infection (p.f.u. per cell)		
	Mengo	VSV	HSV-2
CHO-DHFR 1-8	3×10^{-3}	2.5×10^{-4}	125
CHO-E16 3-17-8	15	2.5×10^{-4}	125

Destruction or protection of the monolayers was recorded 24–36 h after infection. The minimal MOI producing 50% destruction is shown for each virus.

tested. The resistance to Mengo virus varied with multiplicity of infection (MOI) but was clear even at 10 plaque-forming units (p.f.u.) per cell, and after single or multiple virus cycles (Table 1). The inhibition of Mengo virus growth in the amplified CHO-E16 clones appears similar to that observed in IFN-treated cells¹⁴.

In a more severe test of the antiviral state, the CHO-E16 cells were protected against the cytopathic effect (CPE) of Mengo virus, in a wide range of virus concentrations, as are IFN-treated cells. In CHO-DHFR clone 1-8 (or other control clones resistant to MTX), Mengo virus produced complete CPE at typically low MOI, whereas a 5,000-fold MOI was needed to produce CPE in CHO-E16 3-17-8 cells (Table 2). But no protection was seen against VSV or HSV-2 in similar experiments (Table 2). Treatment with rHuIFN- β_1 (1,000 U ml⁻¹) protected all cells against the three viruses (data not shown), confirming that the behaviour of CHO-E16 clones is not due to IFN.

Our studies establish directly that elevation of (2'-5') A synthetase is sufficient by itself, in the absence of IFN, to produce resistance to a picornavirus in CHO cells. This class of virus forms dsRNA intermediates which can activate formation of ppp(A2'p)_nA in infected cells^{15,16} triggering degradation of viral RNA, or more likely ribosomes bound to it^{2,17}, thereby stopping infection. Cell variant studies did not convincingly correlate (2'-5') A synthetase with picornavirus inhibition^{18,19}, possibly due to a failure to measure all four enzyme forms¹⁰, or high 2'-phosphodiesterase²⁰ or low latent RNase in some cells²¹. More IFN-induced proteins could affect picornaviruses (for instance protein kinase²²), which would obscure the correlation whenever cells were treated with IFN. The absence of virus mutants resistant to IFN indeed suggests multiple IFN effects on each virus. Also, the action of IFN appears to be virus-specific and the (2'-5') A synthetase is not sufficient to protect CHO cells against VSV, in line with dissociation between IFN effects on different viruses observed in cell variants^{19,23}. Growth of CHO cells was not prevented by high levels of enzyme, but this could have been due to selection for growth. It will be interesting to

use this direct expression method to study further which IFN activities can be accounted for by the different (2'-5') A synthetases and by the other IFN-induced proteins.

We thank Dr L. Shulman, N. Aloni, Z. Marks, R. Lehrer and P. Federman. Work supported in part by InterYeda, Ltd, Israel.

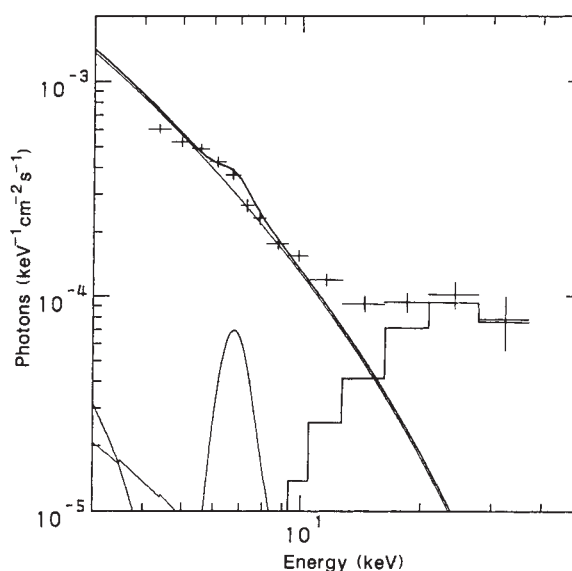
Received 30 July; accepted 23 October 1987.

1. Lengyel, P. A. *Rev. Biochem.* **51**, 251–282 (1982).
2. Revel, M. in *Antiviral Drugs and Interferons: The Molecular Basis of their Activity* (ed. Becker, Y.) 358–434 (Nijhoff, Boston, 1984).
3. Revel, M. & Chebath, J. *Trends biochem. Sci.* **11**, 166–170 (1986).
4. Friedman, R. L., Manly, S. P., McMahon, M., Kerr, I. M. & Stark, G. F. *Cell* **38**, 745–755 (1984).
5. Lerner, A. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 6733–6737 (1984).
6. Samanta, H. *et al. J. biol. Chem.* **261**, 11849–11858 (1986).
7. Staeheli, P., Haller, O., Boll, W., Lindenmann, J. & Weissmann, C. *Cell* **44**, 147–158 (1986).
8. Benesh, P., Mory, Y., Revel, M. & Chebath, J. *EMBO J.* **4**, 2249–2256 (1985).
9. Kerr, I. M. & Brown, R. E. *Proc. natn. Acad. Sci. U.S.A.* **75**, 256–260 (1978).
10. Chebath, J., Benesh, P., Hovanessian, A., Galabru, J. & Revel, M. *J. biol. Chem.* **262**, 3852–3857 (1987).
11. Umlauf, G. & Chasin, L. A. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4216–4220 (1980).
12. Chernajovsky, Y. *et al. DNA* **3**, 297–308 (1984).
13. Revel, M. *et al. Meth. Enzym.* **79**, 149–161 (1981).
14. Falcoff, E., Falcoff, R., Lebleu, B. & Revel, M. *J. Virol.* **12**, 421–430 (1973).
15. Nilsen, T. W., Wood, D. & Baglioni, C. *Virology* **109**, 82–93 (1981).
16. Williams, B. R. G., Golgher, R. R., Brown, R. E., Gilbert, C. S. & Kerr, I. M. *Nature*, **282**, 582–586 (1979).
17. Wreschner, H., James, T. C., Silverman, R. H. & Kerr, I. M. *Nucleic Acids Res.* **9**, 1571–1581 (1981).
18. Lebleu, B. & Content, J. in *Interferon 4* (ed. Gresser, I.) 47–84 (Academic, London, 1982).
19. Kumar, R., Tiwari, R. K., Kusari, J. & Sen, G. J. *J. Virol.* **61**, 2727–2732 (1987).
20. Schmidt, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 4788–4792 (1979).
21. Krause, D. *et al. Eur. J. Biochem.* **146**, 611–620 (1985).
22. Rice, A. P., Duncan, R., Hershey, J. W. B. & Kerr, I. M. *J. Virol.* **54**, 894–902 (1985).
23. Nilsen, T. W., Wood, D. L. & Baglioni, C. *Nature* **286**, 178–180 (1980).
24. Revel, M., Bash, D. & Ruddle, F. H. *Nature* **260**, 139–141 (1976).

Corrigendum

Thermal X-ray emission from supernova 1987A

K. Masai, S. Hayakawa, H. Itoh & K. Nomoto
Nature **330**, 235–236 (1987).



THE above figure should replace Fig. 1 in this paper.